Universidade de Lisboa

Faculdade de Farmácia

Ex vivo expansion of human Tfh-like cells from blood and inflammatory environments

Inês Filipa Pais Monteiro

Dissertation supervised by Dr. Sofia Arriaga Cerqueira and
co-supervised by Professor José Miguel Azevedo

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“Believe in your infinite potential.
Your only limitations are those you set upon yourself.”

Roy T. Bennett, The Light in the Heart

Abstract

Follicular helper T cells (Tfh) are essential components of the adaptive immune system. Interactions between Tfh and B cells play an essential part in humoral immunity, given that this T cell subset is characterized by their B cell helper activity, provision of B cell differentiation signals and formation and maintenance of germinal centers (GCs) within secondary lymphoid organs. Tfh cells have been associated with a variety of diseases, including infectious and inflammatory diseases. Dysregulated Tfh proliferation and cell function have been correlated with contribution to the pathogenesis or poor prognosis of certain autoimmune diseases over the past decade. On the other hand, the presence of Tfh-like cells in breast cancer has also been linked to protective functions. Thus, despite advances in the study of Tfh cell biology, a lack of understanding remains regarding Tfh-like cell subpopulations recently identified in disease conditions.

The present study aimed to expand a PD-1$^+$HLA-DR$^+$ CD4$^+$ Tfh-like subpopulation *ex vivo* in order to define optimal proliferation conditions and functionally analyze expanded cells. Several cell culture conditions were tested, using different concentrations of anti-CD28 and cytokine combinations, to induce differentiation of healthy human CD4$^+$ T cells into PD-1$^+$HLA-DR$^+$ Tfh-like cells. The differentiated population expressed the Tfh markers Bcl-6 and IL-21, confirming a Tfh-like gene signature. Furthermore, expanded cells appeared to display a phenotype relatable to that of Tfh-like subpopulations formerly identified and associated with pathogenic and defensive roles in rheumatoid arthritis and breast cancer, respectively. More specifically, PD-1$^+$HLA-DR$^+$ CD4$^+$ T cells exhibited production of the cytokines IL-10, IFN-γ and TNF-α, as well as the expression of the transcription factors T-bet and Foxp3. These findings suggest the potential of *ex vivo* generated Tfh-like cells to exert functions analogous to resembling cells characterized *in vivo*. The relevance of this study lies in the possibility for *ex vivo* expansion to consist in a useful tool to induce *in vivo* expansion in certain types of cancer, when linked to protective function. Otherwise, this approach allows knowledge extension regarding pathogenic populations emerging from autoimmune diseases such as rheumatoid arthritis.

In conclusion, this work evidences the need to further research Tfh function, presents future directions and provides perspective on factors to be considered that might ultimately contribute to the development of novel therapeutic strategies targeting Tfh-like cells.

Key words: Follicular helper T cells, Tfh cell heterogeneity, cell expansion, functional analysis

Resumo

As células T auxiliares foliculares (Tfh) são um subtipo de células T $CD4^+$ de memória caracterizadas pela expressão de CXCR5, PD-1, ICOS, IL-21 e do seu fator de transcrição chave, Bcl-6, que se localizam principalmente nos centros germinativos (GCs) de órgãos linfoides secundários. No entanto, estas células T $CD4^+$ $CXCR5^+$ também podem ser encontradas no sangue periférico humano, sendo referidas como células Tfh circulatórias (cTfh).

Devido à atividade auxiliar que caracteriza as células Tfh, em conjunto com o fornecimento de sinais de diferenciação a linfócitos B e a sua necessidade para a formação e manutenção de GCs nos órgãos linfoides secundários, as interações entre células Tfh e células B desempenham um papel essencial na imunidade humoral. Desde a sua descoberta, as células Tfh têm sido associadas a diversas doenças, incluindo doenças infeciosas e inflamatórias. A desregulação na proliferação e função celular das células Tfh tem sido correlacionada com a contribuição destas células para a patogénese ou mau prognóstico de certas doenças autoimunes ao longo da última década. Por outro lado, a presença de células semelhantes a Tfh (Tfh-*like*) no cancro da mama também tem sido associada a funções protetoras. Assim, apesar dos avanços no estudo da biologia de células Tfh, ainda existe uma escassez de conhecimento relativamente a subpopulações de células Tfh-*like*.

O presente estudo teve como objetivo expandir uma subpopulação de células Tfh-*like* $CD4^+$ $PD\text{-}1^+HLA\text{-}DR^+$ *ex vivo*, a fim de definir as suas condições ótimas de proliferação e de analisar funcionalmente as células expandidas. Este fenótipo Tfh-*like* foi escolhido devido à prévia evidência de que uma população de células T $CD4^+$ $PD\text{-}1^{hi}$ que co-expressam o complexo principal de histocompatibilidade de classe II (MHC II), onde se insere o marcador de ativação tardia de células T, HLA-DR, se encontravam expandidas no sangue periférico de pacientes com artrite reumatoide soropositiva e a redução dessas células correlacionou-se com uma redução na atividade da doença.

Numa primeira fase, células T $CD4^+$ foram isoladas por separação magnética a partir de amostras de sangue de dadores saudáveis. De forma a induzir a diferenciação das células T $CD4^+$ em células Tfh-*like* $PD\text{-}1^+HLA\text{-}DR^+$, diversas condições de cultura celular foram testadas, utilizando diferentes concentrações de anti-CD28 e diferentes condições de estimulação de citoquinas. Foram testadas as concentrações de 2, 5 e 10 µg/mL de anti-CD28 a fim de alcançar condições adequadas de proliferação para o desenvolvimento de células Tfh-*like*, uma vez que foi demonstrado que o aumento na concentração de anti-CD28 em combinação com uma elevada concentração de anti-CD3 poderia levar ao aumento da ativação de células T. O aumento nos níveis de expressão da molécula PD-1 exibido pelas células T $CD4^+$ de memória $PD\text{-}1^+HLA\text{-}DR^+$ com a adição das citoquinas TGF-β e IL-6 ao meio de cultura, em combinação com um ligeiro aumento na percentagem destas

células, foram fatores determinantes para a seleção da concentração de 5 μg/mL para o anti-CD28. Quando os níveis de expressão de CXCR5 foram medidos nesta condição, foi evidente a escassez de células PD-1$^+$HLA-DR$^+$ CXCR5$^+$ antes e após a cultura celular, pelo que a presença deste recetor não foi considerada uma característica importante para a definição do fenótipo Tfh-*like* expandido. Esta observação está de acordo com estudos previamente realizados em que foram identificadas diversas populações de células Tfh-*like* CXCR5$^-$ em tecidos inflamados não-linfoides.

As condições de estimulação de citoquinas testadas foram baseadas em estudos prévios onde as citoquinas TGF-β, IL-6, IL-12, IL-21, IL-23, IL-27 foram utilizadas para induzir a diferenciação de células com um fenótipo Tfh-*like*. Quando as células T CD4$^+$ foram cultivadas na presença de TGF-β e IL-21 ou TGF-β em conjunto com IL-6 e IL-21, verificou-se uma expansão ligeiramente mais percetível das células PD-1$^+$HLA-DR$^+$. Também foi observada uma tendência para o aumento dos níveis de expressão de PD-1 em comparação com as condições de estimulação sem estas citoquinas presentes. Tendo estes resultados em conta, estas foram as condições de estimulação selecionadas.

Todas as condições de estimulação testadas foram conduzidas na presença da citoquina IL-2. Apesar desta citoquina ser reconhecida como um potente inibidor da diferenciação de células Tfh, comprometendo a manutenção ou sobrevivência de células T que se encontram a divergir na direção da via de diferenciação Tfh, na ausência desta citoquina não foi possível identificar células vivas na população de linfócitos obtida ao fim de 5 dias de cultura celular. Esta observação sugere que as células T CD4$^+$ não sobreviveram num meio deficiente em IL-2. Em adição a esta citoquina, a presença de anti-CD3 e anti-CD28 também foi incluída em todas as condições de estimulação, de forma a mimetizar o processo *in vivo* de ativação de células T onde são necessários a ativação do complexo TCR, do qual proteínas CD3 fazem parte, e sinais de co-estimulação.

Após diferenciação das células nas condições de polarização selecionadas, foi efetuada uma análise à expressão de marcadores de células Tfh, nomeadamente o fator de transcrição repressor Bcl-6 e a citoquina IL-21, confirmando uma assinatura genética semelhante à de células Tfh. Além disso, as células resultantes desta expansão exibiram um fenótipo relacionado com o de subpopulações de células Tfh-*like* recentemente identificadas e associadas a papéis patogénicos e defensivos na artrite reumatoide e no cancro da mama, respetivamente. Mais especificamente, as células T CD4$^+$ PD-1$^+$HLA-DR$^+$ obtidas exibiram produção das citoquinas IL-10, IFN-γ e TNF-α, bem como a expressão dos fatores de transcrição T-bet e Foxp3. Estes resultados, complementados por evidências reportadas em estudos anteriores que relacionam funções das citoquinas analisadas neste trabalho com a artrite reumatoide e/ou o cancro da mama, sugerem o potencial das células Tfh-*like* geradas *ex vivo* para a execução de funções análogas às das células Tfh-*like* caracterizadas *in vivo*. É também importante referir que foi verificada a ausência de relevância estatística relativa às diferentes percentagens de células produtoras de citoquinas obtidas entre todas as condições de estimulação testadas, o que indica que as células expandidas partilham uma única assinatura genética que se traduz também na

partilha de uma única funcionalidade. Esta hipótese foi confirmada pela falta de relevância estatística que também se identificou nos resultados adquiridos na análise à expressão dos fatores de transcrição Bcl-6, T-bet e Foxp3.

Este trabalho contribui positivamente para a área abrangida neste estudo na medida em que foi demonstrado que a expansão *ex vivo* de células Tfh-*like* facilita o estudo de novas subpopulações que surgem em diversas condições de doença. Existe também a possibilidade da expansão *ex vivo* da subpopulação de células Tfh-*like* $CD4^+$ $PD\text{-}1^+HLA\text{-}DR^+$ consistir numa ferramenta de investigação útil com o objetivo de induzir a expansão *in vivo* destas células em certos tipos de cancro, caso estudos futuros permitam relacionar esta população de células a uma função protetora. Caso contrário, esta abordagem experimental poderá permitir o aprofundamento do conhecimento sobre populações patogénicas emergentes de doenças autoimunes, como a artrite reumatoide. No entanto, existe a necessidade de realização de análises fenotípicas adicionais em estudos futuros com vista a confirmar se as células aqui geradas são mais propensas a desempenhar funções associadas a um papel protetor ou detrimentoso.

Em conclusão, este trabalho evidencia a necessidade de investigação adicional relativamente à função de células Tfh para definir a sua absoluta função em contexto de doença. São também apresentadas direções futuras e perspetivas sobre os fatores a serem considerados, de forma a superar limitações do sistema identificadas neste trabalho, e e que podem contribuir para o desenvolvimento de novas estratégias terapêuticas direcionadas às células do tipo Tfh.

Palavras-chave: Células T auxiliares foliculares, heterogeneidade de células Tfh, expansão celular, análise funcional

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Ag	Antigen
APCs	Antigen-presenting cells
Ascl2	Achaete scute-like 2
Bcl-6	B cell lymphoma-6
BFA	Brefeldin A
Blimp-1	B lymphocyte-induced maturation protein-1
BSA	Bovine serum albumin
CCR	CC chemokine receptor 7
CD	Cluster of differentiation
CD40L	CD40 ligand
CEDOC	Chronic Diseases Research Center
cTfh	Circulating follicular helper T cells
CXCL13	Chemokine (CXC motif) ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
ddH2O	Double-distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
ELS	Ectopic lymphoid structures
FACS	Fluorescence-activated cell sorting
FACS-SAP	FACS-Saponin
FBS	Fetal bovine serum
FDCs	Follicular dendritic cells
Fox	Forkhead box
GATA3	GATA-binding protein 3
GCs	Germinal centers
HLA-DR	Human leukocyte antigen DR
ICOS	Inducible T cell co-stimulator
ICOSL	Inducible T cell co-stimulator ligand
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IL-10Rβ	IL-10 receptor subunit beta
Klf2	Krüppel-like factor 2
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex class II
mRNAs	Messenger ribonucleic acids
NACT	Neoadjuvant Chemotherapy
ns	not significant
P/S	Penicillin/Streptomycin
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein-1
PFA	Paraformaldehyde
PLL	Poly-L-lysine
PMA	Phorbol 12-myristate 13-acetate
RNAs	Ribonucleic acids
RORγT	Retinoic acid orphan receptor gamma t
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAP	SLAM-associated protein
SLAM	Signaling lymphocytic activation molecule
SLE	Systemic lupus erythematosus
sRA	Seropositive rheumatoid arthritis
STAT	Signal transducer and activator of transcription
T-bet	T-box factor expressed in T cells
TCR	T-cell receptor
Tfh	Follicular helper T cells
T_{FH}X13	CXCL13-producing Tfh cells
TGF-β	Transforming growth factor bet
Th	Helper T cells
TLS	Tertiary lymphoid structures
T_{PH}	Peripheral helper T cells
Tregs	Regulatory T cells

CHAPTER I • **INTRODUCTION**

1. Immune system

The immune system comprehends a set of mechanisms aimed at protecting the organism against the millions of external threats, such as potential pathogens and toxic or allergenic substances, that it is daily exposed to[1]. To do so, there are two types of immune responses, the innate immunity and the adaptive immunity. The innate immune response consists in the first line of host defense that firstly acts upon distinguishing what belongs to the organism and pathogen features that are external to the host. This type of response is characterized by rapid mechanisms provided by innate immune cells, including macrophages, neutrophils, natural killer cells and dendritic cells, whose goal is to phagocyte the pathogen and generate an inflammatory response, through the release of cytokines and chemokines. In addition, innate immune cells take part in the initiation and direction of adaptive immune response[2,3]. The adaptive immunity has evolved to be more specific, given the variability of antigenic structures and their ability to mutate to avoid host detection. Hence, an increased protective response requires a longer time to display its effects. Adaptive immune response is mainly based on antigen (Ag)-specific receptors expressed on the surface of both T and B lymphocytes, the only cells in the organism with the ability to recognize and specifically respond to each antigenic epitope. These cells mature in the primary lymphoid organs (thymus and bone marrow, respectively), and after that event they initiate adaptive immune responses in secondary lymphoid organs, including lymph nodes and spleen, after presentation of circulating antigens from lymph and blood, respectively. Afterwards, lymphocytes are able to migrate into different body parts in order to exert effector functions. While B lymphocytes relate to an antibody-producing activity, T cells are central to cell-mediated immunity[3,4,5].

1.1. T cell mediated immunity

Lymphocyte mediated immunity takes place when naive cells are activated in an Ag-presenting manner. For that, mature T cells express a T-cell receptor (TCR) comprehending two variable Ag-binding chains non-covalently associated with cluster of differentiation 3 (CD3) proteins forming the TCR complex [5,6]. This receptor is also capable of identifying autoantigens derived from healthy host cells[7]. TCR recognizes a specific Ag presented by antigen-presenting cells (APCs). Antigens are presented in the form of small peptides that are bonded to major histocompatibility complex (MHC) molecules expressed on the surface of APCs. Following peptide-MHC complex recognition by TCR, intracellular signaling is initiated. However, T cell activation through TCR ligation alone is not enough to functionally

activate these cells, as it results in an anergic state. Thus, additional signals are required for productive T cell activation and provided by the interaction of co-stimulatory molecules at the interface between APCs and T cells. Among the cell surface receptors able to enhance signaling through TCR, CD28 has the strongest enhancing capability. This molecule interacts with one of its two ligands, CD80 and CD86, expressed on the surface of APCs[6,7]. Co-stimulatory signals initiate antiapoptotic signals and potentiate IL-2 synthesis by T cells[5]. In addition to TCR signaling and co-stimulation, a third signal consisting in inflammatory cytokines IL-1, IL-12 or interferon alpha/beta (IFN-α/β), was identified as a requirement for optimal T cell activation that culminates in the proliferation of T cells expressing identical receptors that are able to solely recognize the Ag that initiated activation[5,8]. After Ag clearance a drastic decline in the T cell population occurs, and the remaining cells differentiate into memory T cells that acquire an inactive state until re-encounter with the same Ag[5,9].

Among the diversity of T lymphocytes resides an undoubtedly important class of cells: the helper T cells (Th cells). These CD4-expressing ($CD4^+$) T cells provide help for the activation of B lymphocytes to secrete antibodies, for macrophages to destroy digested microbes and for cytotoxic ($CD8^+$) T cells to kill infected target cells[10]. Distinct subsets of $CD4^+$ cells with different cytokine profiles can be identified, namely Th1, Th2, Th9, Th17, Th22, regulatory T cells (Tregs) and follicular helper T cells (Tfh)[5].

2. Follicular helper T cells

Tfh cells were first described as a subset of CXC chemokine receptor 5 ($CXCR5)^+$ T cells in the germinal center (GC) with B helper activity[11]. Nowadays, this subset of memory $CD4^+$ T cells is characterized by the expression of CXCR5, programmed cell death protein-1 (PD-1), inducible T cell co-stimulator (ICOS), interleukin-21 (IL-21) and their key transcription factor, B cell lymphoma-6 (Bcl-6)[12]. In recent years, several studies have shown that the interaction between Tfh and B cells is required for the production of long-lived humoral immunity after infection and vaccination, and also that these cells are necessary for the development and function of GCs[13-15]. An unquestionable importance revolves around Tfh cells, since GCs within secondary lymphoid tissues are essential sites for immunoglobulin (Ig) gene somatic hypermutation as well as maturation and selection of high-affinity B cells to occur[16].

2.1. Tfh cell differentiation

Despite consisting in a complex multistage process, the canonical differentiation of Tfh cells essentially comprehends three phases (Fig. I.1). At an early stage, an Ag-presenting dendritic cell (DC) proceeds to interact with a naive $CD4^+$ T cell and this priming phase occurs in the T cell zone[17,18]. At this point, the fate of the precursor T cell is determined, and that depends on whether the cell upregulated the transcription factor Bcl-6. If so, then the expression of CXCR5 and downregulation of CC chemokine receptor 7 (CCR7), the latter inhibiting T cell migration, is facilitated. Otherwise, Bcl-6-antagonistic transcription factor, B lymphocyte-induced maturation protein-1 (Blimp-1), fails to be repressed and the T cell deviates the Tfh differentiation path to mature into non-Tfh cells, specifically Th1, Th2 or Th17 cells[17,19-21].

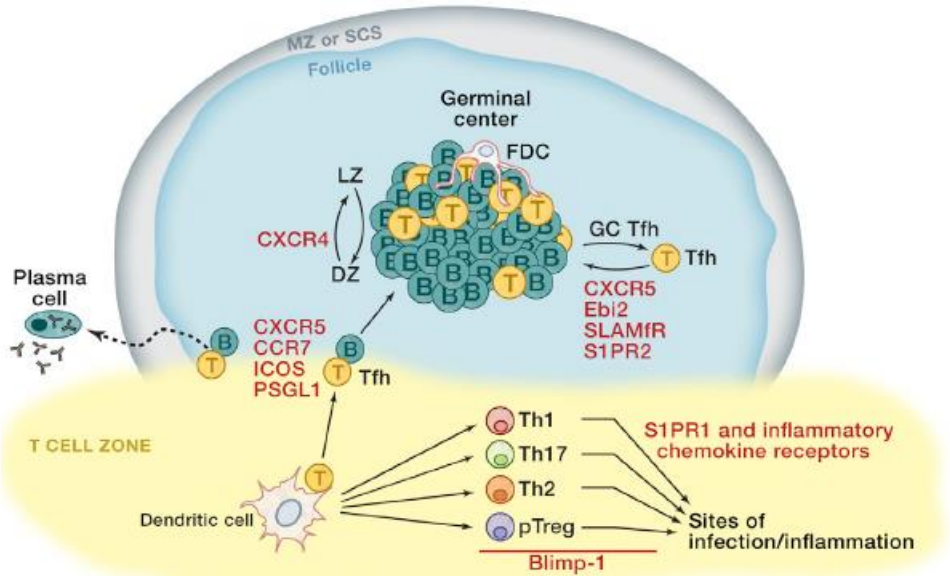


Figure I.1. Multiple stages of Tfh cell differentiation. Upon priming of a naive $CD4^+$ T cell, expression of CXCR5 and downregulation of CCR7 occur, enabling the migration of T cells to the T-B border. After interaction between T and B cells, the germinal center is formed and Tfh cells become fully functional. Migration-related molecules (*red*) are highlighted. From Crotty, S., 2014.

In the second phase, T cells migrate to the T-B border where the interaction with Ag-specific B cells occurs through ICOS/ICOS ligand (ICOSL) signaling and in an Ag-presenting manner. Afterwards, the early committed Tfh cells migrate further into follicles and, in a final stage, the cells fully mature into Tfh cells and the organized microstructure that composes the germinal center is formed, as a result of T-B cell interactions alongside with the presence of follicular dendritic cells (FDCs)[22-26]. GC Tfh cells then proceed to provide B cells with help, classically through IL-21, IL-4, CD40 ligand (CD40L) and chemokine (CXC motif) ligand (CXCL13)[24]. Nevertheless, following differentiation, Tfh cells are not restricted to remaining in the GC, as these cells possess the ability to exit the GC if their help provision is required outside of this structure[17].

The described differentiation process represents the canonical pathway of Tfh cell differentiation. However, since Tfh cells cannot be characterized under a unique phenotype, it is expected that alternative differentiation mechanisms exist as well. This allows the cells to adapt to the different set of environmental conditions and locations that they might be entangled in[17,27].

2.2. Regulation of Tfh differentiation

Each step of Tfh differentiation is influenced by a variety of regulation factors, more precisely by the presence or absence of certain surface receptors, cytokines, transcription factors, among other molecules (Fig I.2). These can either function as positive or negative regulators of Tfh generation, depending on the timing and the signaling pathway they interfere with[22,28].

Tfh cell differentiation depends on the upregulation of the key transcription factor Bcl-6 upon priming of $CD4^+$ T cells. This transcription factor represses the differentiation of other T cell subsets and is also involved in the inhibition of proteins whose downregulation promotes migration to the T-B junction and/or towards the interior of the follicle, and ultimately leads to the induction of CXCR5, ICOS and PD-1 expression[29-34]. Still, in addition to Bcl-6, there are other transcription factors that positively or negatively play a role in the regulation of this complex process of differentiation[35]. From the vast amount of transcription factors involved, c-Maf (responsible for the induction of IL-21 expression)[36], achaete scute-like 2 (Ascl2) and signal transducer and activator of transcription 3 (STAT3) are examples of positive regulators of Tfh formation. Contrariwise, Blimp-1, Krüppel-like factor 2 (Klf2), forkhead box protein O1 (FoxO1) and STAT5 display blockade roles[22,35].

The development of other T cell subsets can be induced by exposure to a single cytokine. For example, T cells differentiate into Th2 effector cells after IL-4 stimulation[37]. However, Tfh cell differentiation is not conducted by the facilitation of a single specific cytokine. Knockout studies in mice demonstrated the critical importance of IL-6 and IL-21 for Tfh cell differentiation[38,39]. The action of IL-27 was likewise studied and confirmed to promote the expression of Tfh markers[40]. In humans, while the role of IL-6 in Tfh differentiation is not clear[24], transforming growth factor beta (TGF-β), IL-12 and IL-23 were shown to be involved in Tfh cell induction[41]. In the presence of IL-12 or IL-23, activated naive human $CD4^+$ T cells displayed increased Tfh cell features, namely CXCR5, ICOS and Bcl-6[42]. TGF-β synergized with IL-12 and with IL-23, activating STAT3 and STAT4 and induced the expression of Tfh cell molecules, including CXCR5, ICOS, IL-21 and Bcl-6[41]. In terms of Tfh blockage, IL-2 is known as a potent suppressor of Tfh cell differentiation through a STAT5-dependent mechanism[43,44]. IL-7 was demonstrated to mediate Bcl-6 repression through activation of STAT5[45], while IL-10 has been suggested to negatively regulate Tfh differentiation in mice, as a lack of signaling through the β subunit of IL-10 receptor (IL-10Rβ) resulted in the increasement of Tfh cell numbers[46].

Along with cytokines, Ag presentation through MHC II and CD28 co-stimulation are critical to directing the differentiation of effector T cells[47,48]. As for surface receptors that promote Tfh cell differentiation, besides the essential ICOS/ICOSL signaling, signaling lymphocytic activation molecule/SLAM-associated protein (SLAM/SAP) and OX40/OX40 ligand (OX40L) signaling also positively regulate Tfh cells[22,49,50].

Posttranscriptional regulation also acts as an important factor, and in this context microRNAs (miRNAs) serve as regulators[51]. miRNAs consist in small noncoding regulatory ribonucleic acids (RNAs) that target messenger RNAs (mRNAs) leading to translational repression, and thus, gene silencing[52]. While the repressor miR-155 positively promotes Tfh cell accumulation during chronic low-grade inflammation, miR-146a counterregulates miR-155, limits Tfh cell accumulation and thus behaves as an inhibitor of Tfh differentiation[53,54].

Many other factors, apart from those mentioned here, influence the differentiation of Tfh cells. The sum of the balancing between all signaling pathways embroiled in this multistep process determine whether the formation of Tfh cells manages to be fully completed.

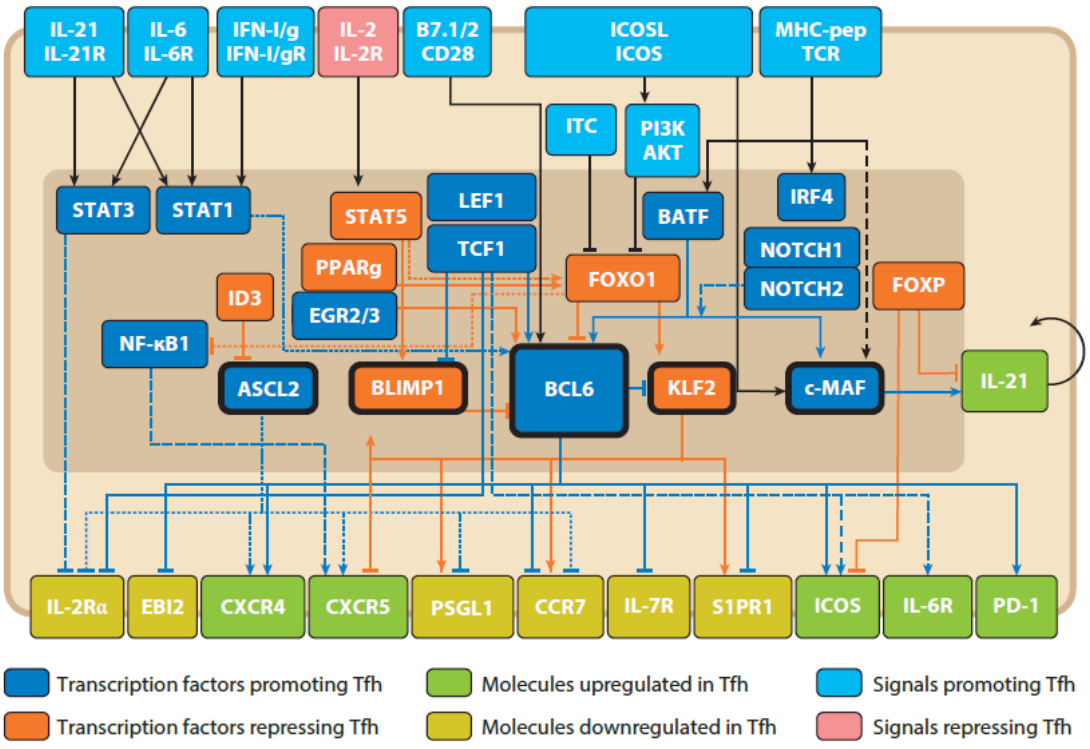


Figure I.2. Regulators of Tfh cell differentiation. Among the considerable variety of regulation factors, this figure illustrates the connection between several signaling pathways and transcription factors that promote or inhibit Tfh differentiation. From Vinuesa, C.G. *et al*., 2016.

2.3. Circulating Tfh cells

Tfh cells are primarily localized in the B cell follicles, specifically in the GCs of secondary lymphoid tissues, as a result of the expression of CXCR5 that allows their migration in response to ita specific ligand CXCL13, expressed by FDCs located in the light zone of the GC[15,55]. However, these $CXCR5^+$ $CD4^+$ T cells can also be found in human peripheral blood and are commonly referred to as circulating Tfh (cTfh)[56]. The relationship between them and GC Tfh cells remains uncertain[57]. The phenotype of these two subsets of cells is notably distinct, since it has been demonstrated that the circulating counterpart of GC Tfh cells do not express Bcl-6[56,58]. Furthermore, these blood cTfh cells have been shown to express lower levels of PD-1 and ICOS than tissue Tfh cells[58]. It was believed that the majority of circulating $CXCR5^+$ cells consist in resting memory Tfh cells[59], still, recent research identified activated cTfh cell populations in peripheral blood[60]. One study revealed a subset of $ICOS^+$ $CD38^+$ cTfh cells that contained Ag-specific cells post influenza vaccination, while $ICOS^-$ $CD38^-$ cells presented stable memory[61]. In terms of their function, it was shown that cTfh cells are composed of distinct subsets with singular phenotype and function, namely Th1, Th2 and Th17 cells, with different abilities to regulate B cell responses (Fig. I.3). While Th2 and Th17 cells within $CXCR5^+$ compartment successfully helped naive B cells in the production of immunoglobulins via IL-21, Th1 cells lacked this helper B cell capacity. The division between the three subsets is defined by the differential expression of the chemokine receptors CXCR3 and CCR6[62,63].

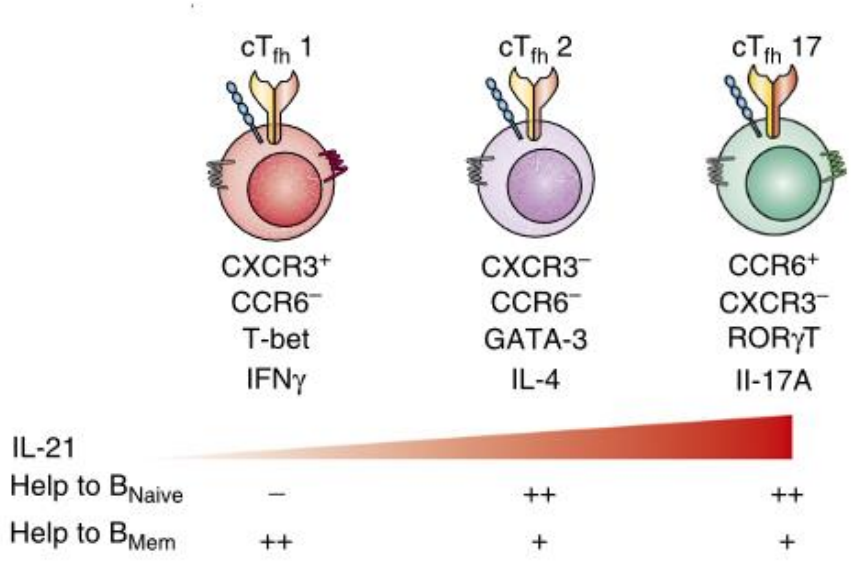


Figure I.3. Distinct subsets of circulating Tfh cells. According to their CXCR3 and CCR6 expression, cTFh cells can be divided into three distinct subsets: cTfh1, cTfh2 and cTfh17. These subsets display differences in their ability to provide help to B cells. From Koutsakos, M. *et al*., 2019.

2.4. Tfh cells in human health

Tfh-B cell interactions are crucial for the normal function of humoral immunity, considering that these interactions allow the production of high affinity antibodies upon the occurrence of somatic hypermutation that takes place within the GCs, during natural infection or following vaccination. Not only do Tfh cells play an important role inducing differentiation of B cells into plasma cells, they also grant B cells with different signals that provide cell survival and proliferation, as well as stimulation of somatic hypermutation and signals that will culminate in the death of inappropriate B cells. Therefore, it is essential that the processes involved in the interactions between these two types of cells remain strongly regulated to avoid the development of autoimmunity or lymphoid malignancy[13,15,24,64]. Furthermore, since Tfh cells are involved in many immune responses-associated scenarios and their differentiation can either be positively or negatively regulated, it is important to fully understand the processes involved in the generation of these cells. In this manner, new treatment strategies directly targeting Tfh cell manipulation might be developed.

It has been recently reported that cTfh1 cells play a fundamental role for the generation of antibody response following influenza vaccination containing inactivated viral components. However, influenza vaccines have been displaying poor effectiveness in producing long-lasting immunity, for example as low as 10% in 2013–2014 and 7% in 2014–2015 for the H3N2 subtype, and this is due to a lack of high quality antibody response[24,65,66]. A deeper knowledge of the role of Tfh cells in the generation of highly affinity matured antibody responses and overall of the immune mechanisms that induce immunological memory post-vaccination is necessary in order to improve vaccination strategies that promote long-lasting immune protection[65].

2.5. Tfh cells and disease

Dysfunctional regulation of Tfh-related processes, such as differentiation or proliferation, might lead to a disease status as a result of the essential role these cells play in protective immunity against pathogens. Deficiency of Tfh cells is usually associated with immunodeficiency, whereas an excessive amount of Tfh cells supports the development of autoimmunity[67]. Ever since their identification, Tfh cells have been related to a wide range of diseases (Fig. I.4). A great amount of research has been conducted, and recent studies show that Tfh cells display both protective or pathogenic roles in a vast diversity of diseases, including autoimmune diseases and several types of cancers[24]. Notwithstanding, these cell populations consist in Tfh-like cells that do not display the exact same phenotype as *bona fide* Tfh cells.

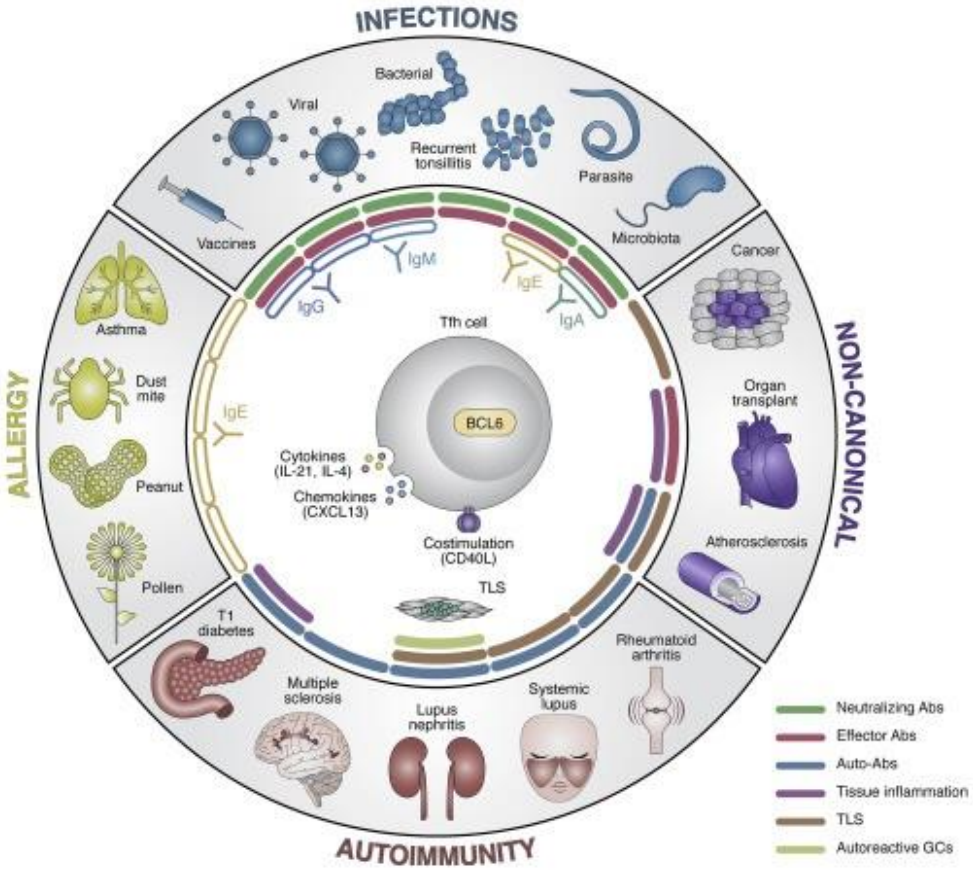


Figure I.4. Relationships of Tfh cells to diseases. Recent evidence has been revealing the pathogenic or protective contribution of Tfh cells to multiple disease conditions, including autoimmune diseases and several types of cancer. From Crotty, S., 2019.

2.5.1. Autoimmune diseases

Earlier this year, a novel $CD4^+$ T cell population resembling Tfh cells, described as $CXCR5^-CXCR3^+PD\text{-}1^{hi}$ cells that lacked the expression of IL-21 and CXCL13, was found to be expanded in the blood of systemic lupus erythematosus (SLE) patients and suggested to contribute to the pathogenesis of this autoimmune disorder through a mechanism involving IL-10 and succinate that provided help to B cells[68]. Another $CXCR5^-PD\text{-}1^{hi}$ $CD4^+$ T cell population, with high expression levels of ICOS and MHC II, was recently identified in rheumatoid arthritis. These cells, predominantly expanded in the synovial fluid and inflamed tissues from patients with seropositive rheumatoid arthritis (sRA), exhibited a Tfh-associated gene profile linked with B cell helper function[69]. In addition to these data, others had already described expanded Tfh-like cells in peripheral blood of individuals suffering from SLE and diverse autoimmune disorders such as Sjögren's syndrome and juvenile dermatomyositis[58,62,70,71].

2.5.2. Cancer

Whilst it comes as no surprise that Tfh cells are associated with autoimmune diseases, evidence that these helper cells are relevant in several types of human cancer in a protective manner was rather unpredictable[24]. In both breast cancer and colorectal cancer, a protective immunity action has been associated with the presence of a Tfh gene profile[72,73]. Particularly in breast cancer, Tfh gene signature was correlated with long-term survival of patients[72]. Furthermore, recent data identified an infiltrating CXCL13-producing CXCR5$^-$PD-1^{hi} CD4$^+$ T cell subpopulation and suggested that these Tfh-like cells act as B cell attractors and guide their migration with posterior formation of tertiary lymphoid structures (TLS) at the tumor site[74]. These structures represent ectopic lymphoid organs in the inflamed tissue, and their presence could provide a microenvironment to the tumor tissue enabling the recruitment of other immune cells with antitumor immunity[24,75]. Comparable cells were observed in melanoma tumors[76].

2.6. Tfh-like cells

As discussed above, Tfh-like cell populations identified in several disease conditions are not phenotypically identical to GC Tfh cells. It was showed that GC Tfh cells do not express T-box factor expressed in T cells (T-bet), GATA-binding protein 3 (GATA3), retinoic acid orphan receptor gamma t (RORγt), Foxp3 and only produced limited cytokines related to different Th cell subsets. Moreover, key molecules involved in the development, migration and function of Tfh cells were identified by gene-expressing profiling, i.e., *BCL6*, *ASCL2*, *IL21*, *PDCD1* (PD-1) and *ICOS*[22,77]. On the other hand, Tfh-like cells are not defined by a strict phenotypic and transcriptional profile. Opposed to typical cTfh cells described in the literature, plenty of the identified Tfh-like cell populations lacked CXCR5[78]. Although it was described that deficiency in CXCR5 translated into absence of B helper activity[62], controverting data aforementioned suggested the involvement of CXCR5$^-$ Tfh-like cells in the pathogenesis or negative mediation of autoimmune disorders as well as the correlation with a protective role in cancer. A recent review compared the two CXCR5$^-$PD-1^{hi} CD4$^+$ T cell populations mentioned here, namely CXCL13-producing Tfh (T_{FH}X13) and peripheral helper T (T_{PH}) cells, found in breast cancer and rheumatoid arthritis, respectively[79]. Both cell populations were described as functionally and phenotypically resembling of typical Tfh cells lacking CXCR5, despite also expressing molecules uncorrelated with Tfh cells, such as the transcription factors T-bet and Foxp3, involved in the development of Th1 and Tregs, respectively[80,81] (Fig. I.5). Regardless of their similarities, T_{FH}X13 cells were related to a defender action in cancer while the T_{PH} subpopulation was linked to increased autoimmune disease severity.

In sum, a scarcity of understanding regarding the wide range of Tfh cell subpopulations embroiled in the context of human disease prevails and represents an obstacle to, first: the absolute definition of their role and clinical significance in illness conditions; and second: the

development of new therapeutic approaches against the wide range of diseases Tfh cells have been associated with.

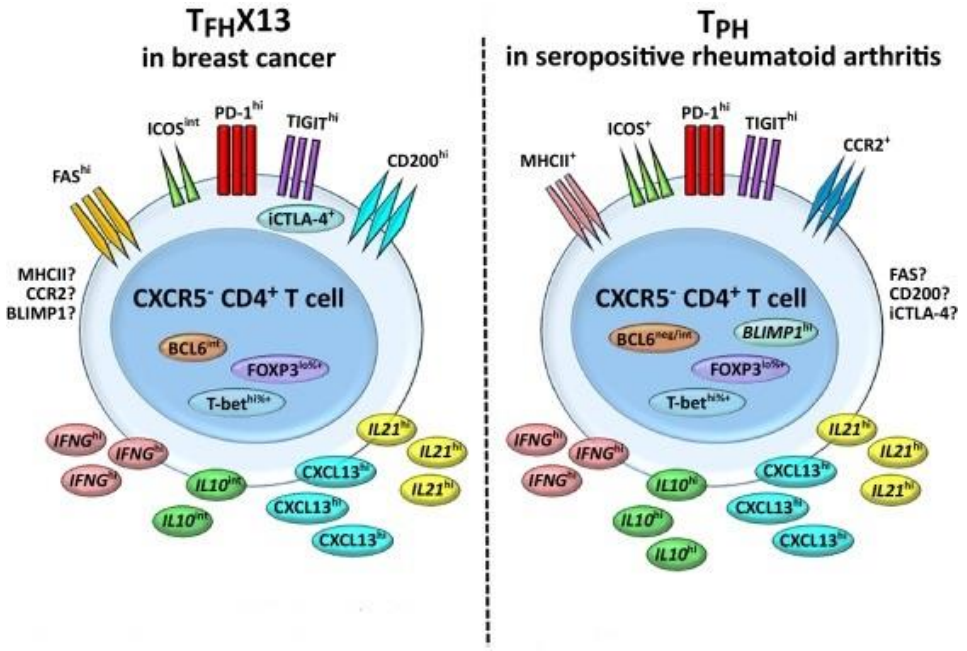


Figure I.5. Protective and pathogenic Tfh-like cell populations. Phenotypically similar CXCR5$^-$PD-1^{hi} CD4$^+$ T cells associated with contradictory roles in breast cancer and seropositive rheumatoid arthritis. From Gu-Trantien, C. and Willard-Gallo, K., 2019.

3. Objectives of the study

Although a lot of research has been conducted in Tfh cell biology in human health and disease, there is still a lack of knowledge hindering the definition of Tfh cell roles in diseases. Furthermore, recent data revealed novel Tfh-like gene signatures displayed in CD4$^+$ T cells in inflamed tissues and peripheral blood of patients, as presented above, reinforcing the need to further study the emerging subpopulations of Tfh-like cells that exhibit pathogenic or protective functions in disease.

The present work intended to achieve the *ex vivo* expansion of a Tfh-like cell subpopulation of PD-1$^+$HLA-DR$^+$ CD4$^+$ T cells from blood samples, given that previous research associated Tfh-like cells expressing both PD-1 and MHC II with disease severity in rheumatoid arthritis[69], suggesting that this cell population might be of clinical significance. To this end, CD4$^+$ T cells were isolated from blood samples of human healthy donors and stimulated *ex vivo* under different conditions, in order to induce differentiation into Tfh-like cells. The expansion of PD-1$^+$HLA-DR$^+$ CD4$^+$ Tfh-like cells was evaluated by flow cytometry and further functional analysis of the expanded population was posteriorly performed.

Ultimately, this approach will contribute to define the optimal proliferation conditions of this Tfh-like cell subpopulation, which might be a useful tool to induce its expansion *in vivo*, in order to control certain types of cancer. On the other hand, *ex vivo* expansion of Tfh-like cells will allow a deeper study and characterization of its pathogenic population counterparts that arise in inflammatory diseases.

This work was inserted in the project “Reprogramming inflammatory-follicular T cells (Tfhi): from the clinic to precision immunotherapies”, funded by FCT – Fundação para a Ciência e a Tecnologia (02/SAICT/2017/029520).

CHAPTER II • **MATERIAL AND METHODS**

1. Materials

1.1. Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) was supplemented with 10% fetal bovine serum (FBS; Merck) and 1% Penicillin/Streptomycin (P/S; Thermo Fisher Scientific). Biocoll (1.077 g/mL) used in density gradient cell separation was acquired from Merck. The freezing medium used in cryopreservation of peripheral blood mononuclear cells (PBMCs) contains 90% FBS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich). The MojoSort™ Human CD4 T Cell Isolation Kit was from Biolegend. The 5X MojoSort buffer from this kit consists of 5X phosphate-buffered saline (PBS; VWR), 10 mM ethylenediamine tetraacetic acid (EDTA) and 25 mg/mL bovine serum albumin (BSA). Fluorescence-activated cell sorting (FACS) buffer is composed of 1X PBS and 2% FBS. FACS-Saponin (FACS-SAP) buffer is constituted by FACS buffer and saponin 0.1% (Carl Roth). Paraformaldehyde (PFA) used to fixate cells was from Sigma-Aldrich. For intracellular staining for transcription factors, eBioscience™ Foxp3/Transcription Factor Staining Buffer Set obtained from Thermo Fisher Scientific was used. For cytokine production, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and ionomycin (Merck) were used as well as Brefeldin A (BFA; Sigma-Aldrich).

1.2. Antibodies

Table II.1. Antibodies used for staining and cell stimulation

Antibody	Isotype	Supplier	Reference	Fluorochrome	Final concentration
CD4	Mouse IgG1	Biolegend	300530	PerCP-Cy5.5	2 µg/mL
CD62L	Mouse IgG1	Biolegend	304803	FITC	4 µg/ml
PD-1	Mouse IgG1	Biolegend	329918	PE-Cy7	4 µg/mL
	Mouse IgG1	Biolegend	329920	BV421	1 µg/mL
HLA-DR	Mouse IgG2a	Biolegend	307606	PE	0.8 µg/mL
CXCR5	Mouse IgG1	Biolegend	356926	APC-Cy7	4 µg/mL
IL-21	Mouse IgG1	Biolegend	513006	A647	1 µg/mL
IL-10	Rat IgG1	Biolegend	501419	PE-Cy7	0.2 µg/mL
IFN-γ	Mouse IgG1	Biolegend	502544	BV510	2 µg/mL
TNF-α	Rat IgG1	Biolegend	506322	PerCP-Cy5.5	1.3 µg/mL

Bcl-6	Rat IgG2a	Biolegend	358512	PE-Cy7	0.5 µg/mL
T-bet	Mouse IgG1	BD Pharmingen™	561264	A647	1:50
Foxp3	Mouse IgG1	Biolegend	320011	A488	1.2 µg/mL
anti-CD3	Mouse IgG1	Biolegend	300402	-	5 µg/mL
anti-CD28	Mouse IgG1	Biolegend	302914	-	2, 5,10 µg/mL
anti-IL-4	Mouse IgG1	BD Pharmingen™	559062	-	10 ng/mL
anti-IFN-y	Mouse IgG1	Immunotools	21853530	-	10 ng/mL

1.3. Dyes

Table II.2. Dyes used to exclude dead cells in FACS analysis

Dye	Supplier	Fluorochrome
Fixable Viability Dye eFluor™ 506	Invitrogen	BV510
Fixable Viability Dye eFluor™ 780	Invitrogen	APC-Cy7

1.4. Cytokines

Table II.3. Cytokines used during cell culture

Cytokine	Supplier	Final concentration
IL-2	NIH AIDS Reagent Program	20 IU/mL
TGF-β	ImmunoTools	10 ng/mL
IL-6	ImmunoTools	10 ng/mL
IL-12	ImmunoTools	10 ng/mL
IL-21	ImmunoTools	10 ng/mL
IL-23	PeproTech	10 ng/mL
IL-27	ImmunoTools	10 ng/mL

2. Methods

2.1. Blood sample isolation of CD4⁺ T cells

2.1.1. Isolation of peripheral mononuclear cells from blood samples

Whole blood was collected from adult healthy donors at CEDOC by certified staff. Approval for this protocol was granted by the ethics committee of NOVA Medical School of Lisbon. Written informed consent was obtained from all donors.

PBMCs were isolated with Ficoll density gradient separation, in which whole blood diluted in a ratio 1:2 with PBS 1X was carefully deposited on top of Ficoll (1:3) and centrifuged at 1200 x g for 30 minutes without brake. After collecting the PBMCs ring, cells were washed twice with PBS 1X at 700 x g for 10 minutes. PBMCs were cryopreserved in freezing medium containing 90% FBS and 10% DMSO, the first 48 hours at -80°C and then at -150°C until use.

2.1.2. Isolation of CD4⁺ T cells

PBMCs were thawed and cultured overnight in supplemented RPMI medium with 20 IU/mL IL-2 (Table II.3) at 37°C, 5% CO_2. $CD4^+$ T cells were then isolated by negative selection using the MojoSort™ Human CD4 T Cell Isolation Kit and following the MojoSort™ Isolation Kits No Wash Protocol. Cells at $2x10^6$ cells/mL in supplemented RPMI medium were rested for 2 hours at 37°C, 5% CO_2.

2.2. Tfh-like cell culture

$CD4^+$ T cells were cultured at $2x10^6$ cells/mL for 5 days at 37°C, 5% CO_2 in supplemented RPMI with IL-2 (20 IU/mL) under stimulation with plate-bound 5 µg/mL anti-CD3 and 2, 5 or 10 µg/mL anti-CD28 antibodies. 96 round U-bottomed plates used were coated with poly-L-lysine (PLL; Sigma-Aldrich) at 2 µg/mL. The following cytokines were added to generate Tfh-like subpopulation: TGF-β, IL-6, IL-12, IL-21, IL-23, IL-27 (Table II.3); each at 10 ng/mL. In some experiments neutralizing 10 ng/mL anti-IL-4 and 10 ng/mL anti-IFN-γ antibodies were added (Fig. II.1). At day 3 of cell culture, supplemented RPMI medium with 20 IU/mL IL-2 and 10 ng/mL of the stimulating cytokines and neutralizing antibodies was renewed.

2.3. Cytokine production

After 4 days in culture, cells were stimulated with 50 ng/mL PMA and 0.5 µg/mL ionomycin and treated with 2 µg/mL BFA, for 16 hours. PMA, which activates protein kinase C, synergizes with the calcium ionophore ionomycin to bypass TCR-mediated processes, activating several intracellular signaling pathways and resulting in T lymphocyte activation and cytokine production[82,83]. BFA functions as an inhibitor of intracellular protein transport

between the endoplasmic reticulum and the Golgi apparatus, leading to aggregation of cytokines within the cells[84].

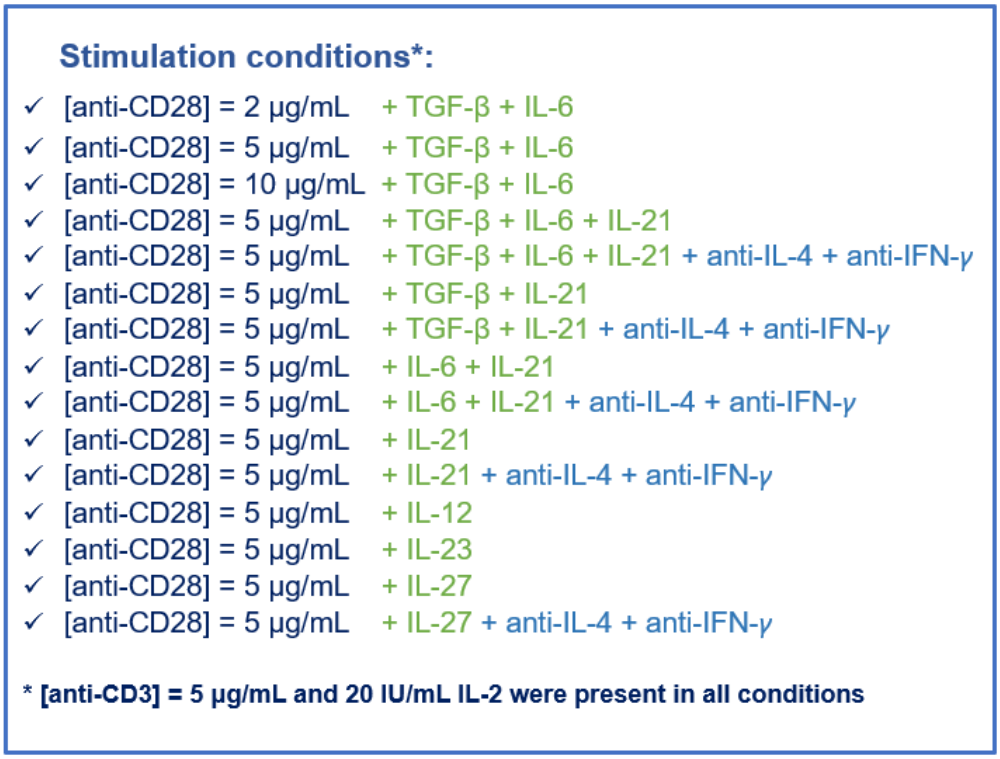


Figure II.1. *Ex vivo* differentiation of CD4⁺ T cells into Tfh-like cells. Stimulation conditions in which CD4⁺ T cells were cultured for 5 days in addition to anti-CD3 and IL-2.

2.4. Flow cytometry

For surface staining, cells were washed twice with PBS 1X at 500 x g for 3 minutes and stained with either Fixable Viability Dye eFluor™ 506 or Fixable Viability Dye eFluor™ 780 (Table II.2) in the dark (as all incubations were performed), for 20 minutes on ice. Then, cells were washed twice with FACS buffer and incubated with the primary surface fluorochrome-conjugated antibodies (Table II.1) for 20 minutes on ice. After washing twice with FACS buffer, for cytokine analysis, cells were fixed with 1% PFA at room temperature (RT) to avoid precipitates, for 20 minutes, and washed once with FACS buffer. For analysis of cytokine production, membrane permeabilization was performed with FACS-SAP for 20 minutes at RT. Cells were centrifuged at 500 x g for 3 minutes and posteriorly incubated with primary intracellular fluorochrome-conjugated antibodies (Table II.1) diluted in FACS-SAP for 30 minutes at RT. After staining, cells were washed once with FACS-SAP and then with FACS buffer. To asses transcription factors expression, after surface labeling, the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set was used. Thus, fixation and

permeabilization were performed for 30 minutes at RT. Cells were then centrifuged at 500 x g for 3 minutes and the intracellular staining was performed with primary intracellular fluorochrome-conjugated antibodies (Table II.1) for 30 minutes at RT. Lastly, cells were washed twice with permeabilization buffer from the kit in a 1:10 dilution of double-distilled water (ddH_2O) and once with FACS buffer. Data was acquired with a FACS Canto II flow cytometer from BD Biosciences and analyzed using FlowJo (BD Life Sciences).

2.5. Statistical analysis

Statistical significance was determined using the Wilcoxon matched-pairs test, with p-values of $< 0,05$ considered statistically significant. Statistical analysis was performed in the software GraphPad Prism 6 (GraphPad Software, Inc.)

CHAPTER III • **RESULTS**

1. *Ex vivo* expansion of Tfh-like cells

1.1. Definition of the gating strategy applied in the identification of PD-1⁺HLA-DR⁺ CD4⁺ T cells

Previous studies conducted the induction of Tfh cell differentiation in peripheral CD4⁺ T cells. In addition to CD3 and CD28 stimulation, required for the activation of T cells[6], cell culture was managed in the presence of cytokines, including TGF-β, IL-6, IL-12, IL-21, IL-23 and IL-27[42,85-87]. Here, different concentrations of anti-CD28 and different combinations of the mentioned cytokines were tested. Additionally, the neutralizing antibodies anti-IL-4 and anti-IFN-γ were tested as well, due to their previous use in *in vitro* culture of Tfh-like cells[85].

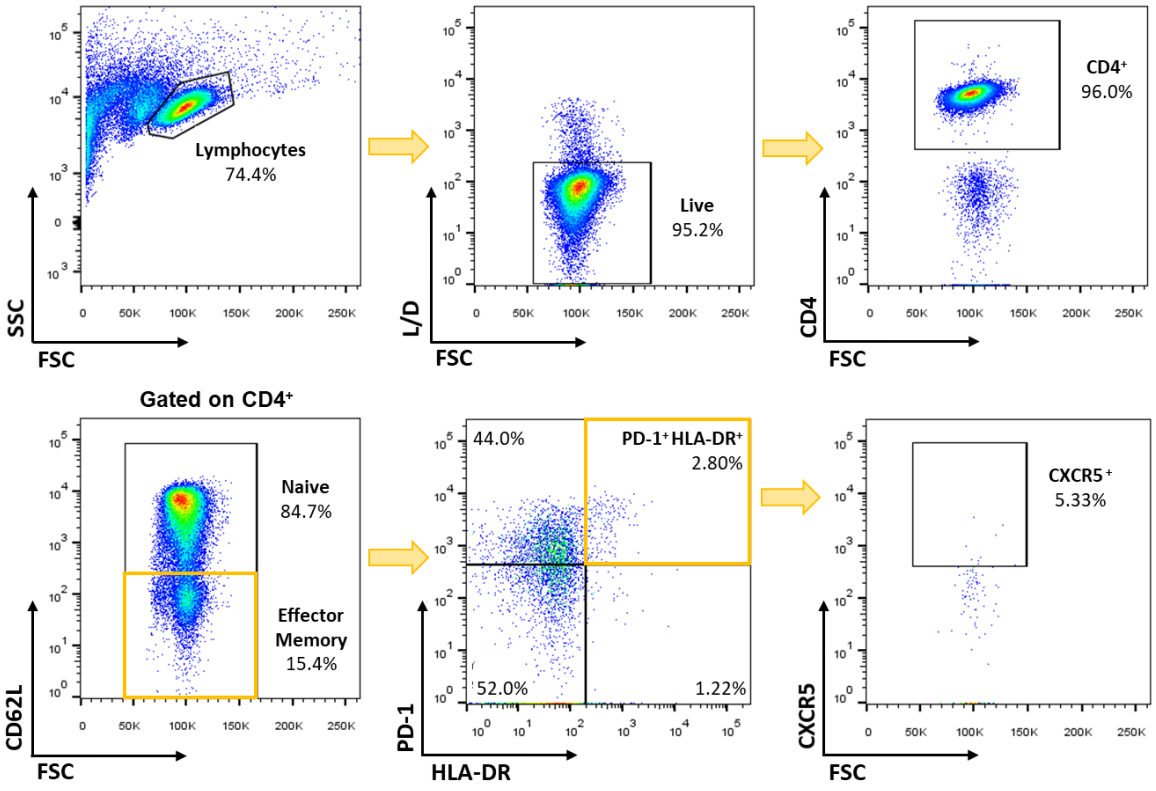


Figure III.1. Representative flow cytometry results of CD4⁺CD62L⁻PD1⁺HLA-DR⁺ Tfh-like cells before stimulation. Human PBMCs were isolated from blood samples of healthy donors and CD4⁺ T cells were posteriorly purified. Cells were then stained with Fixable Viability Dye eFluor™ 506, followed by staining with antibodies against the analyzed cell surface markers. Flow cytometry data was acquired in BD FACS Canto II.

For the identification of Tfh-like cells, the lymphocyte population was firstly defined as previously explained in Adan, A. *et al*. (2017)[88]. After selecting the live cells, $CD4^+$ T cells were identified. As expected, these cells represented the majority of live cells, since $CD4^+$ T cells were previously purified by magnetic separation from isolated PBMCs (chapter II, section 2.1.2). Gated cells were then divided into naive and effector memory cells, according to their CD62L expression, which enables the distinction between those two types of T cells[89]. Only $CD62L^-$ cells were selected, since Tfh cells were demonstrated to down-regulate the expression of CD62L[90], and therefore our population of interest was included in the effector memory cell population. In addition to PD-1, one of the key molecules highly expressed in Tfh cells[91], human leukocyte antigen DR (HLA-DR), consisting in the class II MHC protein with the highest expression level on APCs in most humans[92] and recognized as a late T cell activation marker[93-95], was also included in gating strategy. Notably, it was previously reported that PD-1^{hi} $CD4^+$ T cells co-expressing MHC II were expanded in the peripheral blood of seropositive rheumatoid arthritis patients and the reduction of those cells after treatment escalation correlated with a reduction in disease activity[69]. Moreover, higher expression of HLA-DR in cytotoxic T lymphocytes was recently found to be positively associated with response to neoadjuvant chemotherapy (NACT) in breast cancer, while lower levels of HLA-DR expression in Tregs displayed a negative correlation with NACT response[96]. These data suggest that the activation state of T lymphocytes might influence their pathogenic or protective role in the diseases these cells are associated with. Lastly, CXCR5, the defining marker of Tfh cells[97], was also measured. This molecule is expressed in cTfh cells from human peripheral blood and also in Tfh-like cell populations expanded *in vitro* from $CD4^+$ T cells[42,56,85,87].

The frequency of the mentioned surface markers was measured in $CD4^+$ T cells isolated from peripheral blood of healthy donors, before cytokine stimulation (day 0), as displayed in figure III.1. This measurement allows the assessment of Tfh-like cell expansion after 5 days of culture in polarizing conditions.

1.2. PD-1^+HLA-DR^+ $CD4^+$ T cell expansion in the presence of TGF-β and IL-6 is influenced by CD28 co-stimulation

As formerly demonstrated, the use of different anti-CD28 concentrations in combination with a high concentration of anti-CD3 might have an impact on T cell activation[98] which in turn could negatively influence Tfh-like cell development. To test this, peripheral $CD4^+$ T cells were cultured under the same CD3 stimulation conditions and varying concentrations of anti-CD28 for 5 days. PD-1^+ HLA-DR^+ $CD4^+$ T cells were then identified following the defined gating strategy (Fig. III.2) and the expansion of this population was evaluated, by analyzing the fold change in the percentage of PD-1^+HLA-DR^+ $CD4^+$ T cells, gated on $CD62L^-$, at day 5 comparing to day 0. In the presence of 2 and 10 µg/mL of anti-CD28, the increase in this cell population was slightly higher, as reflected by the median fold change

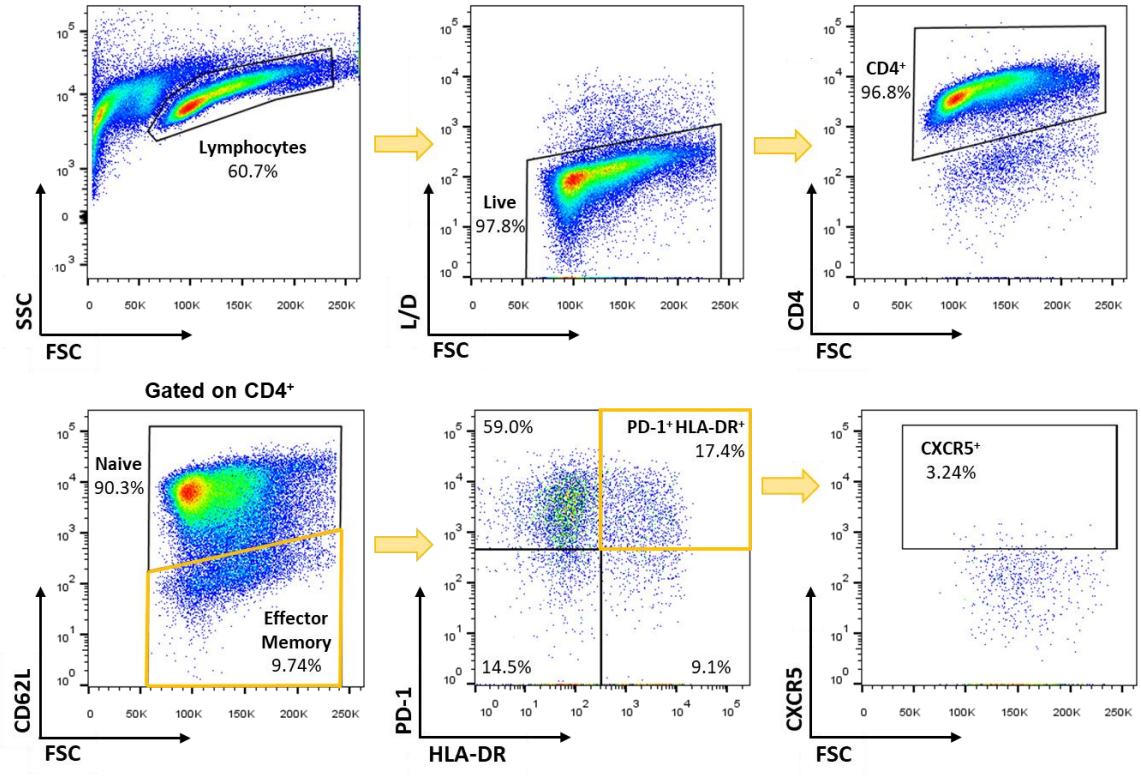


Figure III.2. Representative flow cytometry results of CD4$^+$CD62L$^-$PD1$^+$HLA-DR$^+$CXCR5$^+$ Tfh-like cells after stimulation in polarizing conditions. Purified CD4$^+$ T cells were cultured under CD3 and CD28 stimulation in the presence of TGF-β and IL-6 for 5 days. Cells were then stained with Fixable Viability Dye eFluor™ 506, followed by staining with antibodies against the analyzed cell surface markers. Flow cytometry data was acquired in BD FACS Canto II.

(Fig. III.3A, B, C). However, in the presence of the cytokines TGF-β and IL-6, the expansion of PD-1$^+$HLA-DR$^+$ cells only occurred at 5 and 10 µg/mL anti-CD28. This increase was somewhat higher at 5 µg/mL anti-CD28. Furthermore, the analysis of the fold change of the mean fluorescence intensity (MFI) from both PD-1 and HLA-DR molecules shows that cytokine addition resulted in a significant increment of PD-1 expression in the expanded cell population at 2 and 5 µg/mL anti-CD28. No relevant changes in HLA-DR MFI values were observed in neither anti-CD28 concentrations. Taking these results into account, further research in regard to the definition of optimal Tfh-like cell proliferation was managed with the inclusion of 5 µg/mL anti-CD28 in cell culture.

1.3. PD-1$^+$HLA-DR$^+$ CD4$^+$ T cells express low levels of CXCR5

Next, the percentage of PD-1$^+$HLA-DR$^+$ T CD4$^+$ T cells that expressed CXCR5 was assessed for the selected anti-CD28 concentration. As demonstrated in figure III.4, and despite the presence of two outliers, following 5 days of cell culture, CXCR5 expression

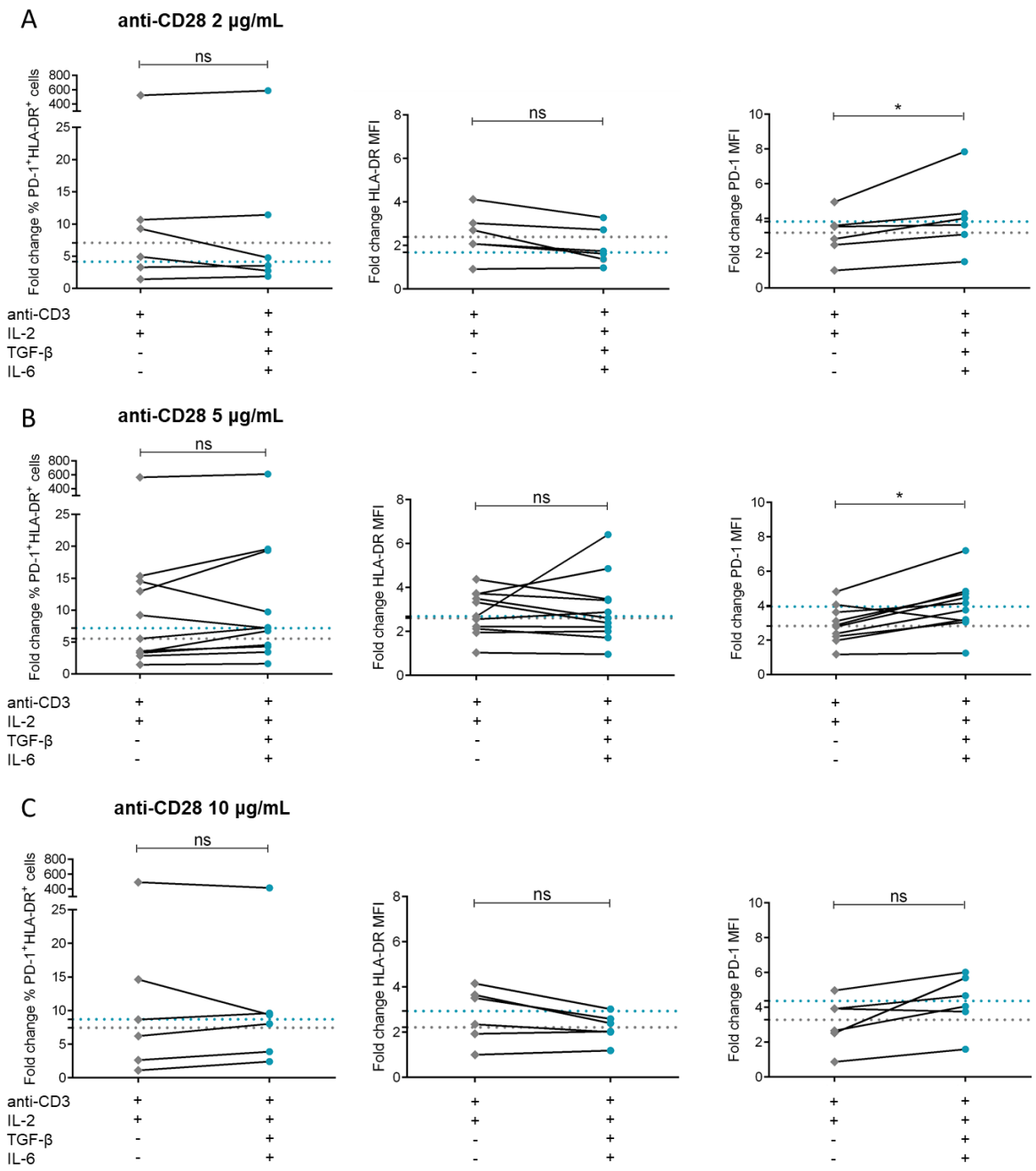


Figure III.3. Influence of different anti-CD28 concentrations in PD-1⁺HLA-DR⁺ CD4⁺ T cell expansion. Human CD4⁺ T cells were cultured under CD3 stimulation and the indicated concentration of anti-CD28, in the presence or absence of TGF-β and IL-6 for 5 days. Fold change of PD-1⁺HLA-DR⁺ cell percentages and MFI of HLA-DR (middle) and PD-1 (right) corresponding to anti-CD28 at **(A)** 2 µg/mL, **(B)** 5 µg/mL and **(C)** 10 µg/mL in the presented stimulation conditions. Each data point represents an individual donor. Grey dashed lines represent the medians in the absence of TGF-β and IL-6 while blue dashed lines represent the medians in the presence of TGF-β e IL-6. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant; *p < 0.05).

significantly decreased (median 3.28%) from the already small percentage of CXCR5$^+$ cells (median 6.38%). Given the fact that most PD-1$^+$HLA-DR$^+$ cells lacked the classical Tfh cell-defining marker CXCR5, and that CXCR5$^-$ Tfh-like cells were found in autoimmune diseases and breast cancer[68,69,74], as discussed in sections 2.5.1 and 2.5.2 of chapter I, the expression of this receptor was not considered as a key feature for the definition of the expanded Tfh-like phenotype.

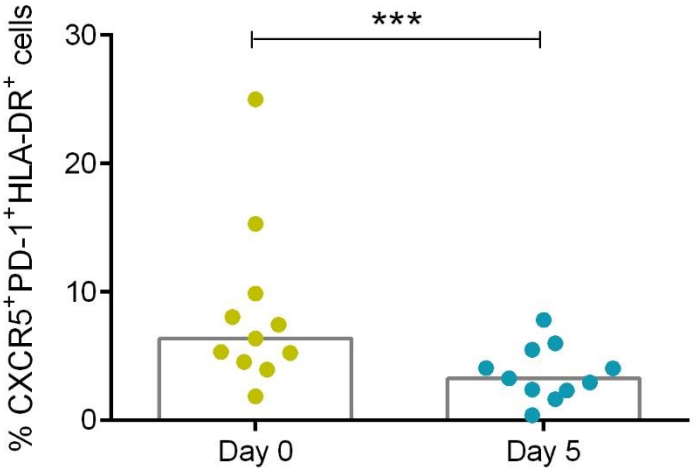
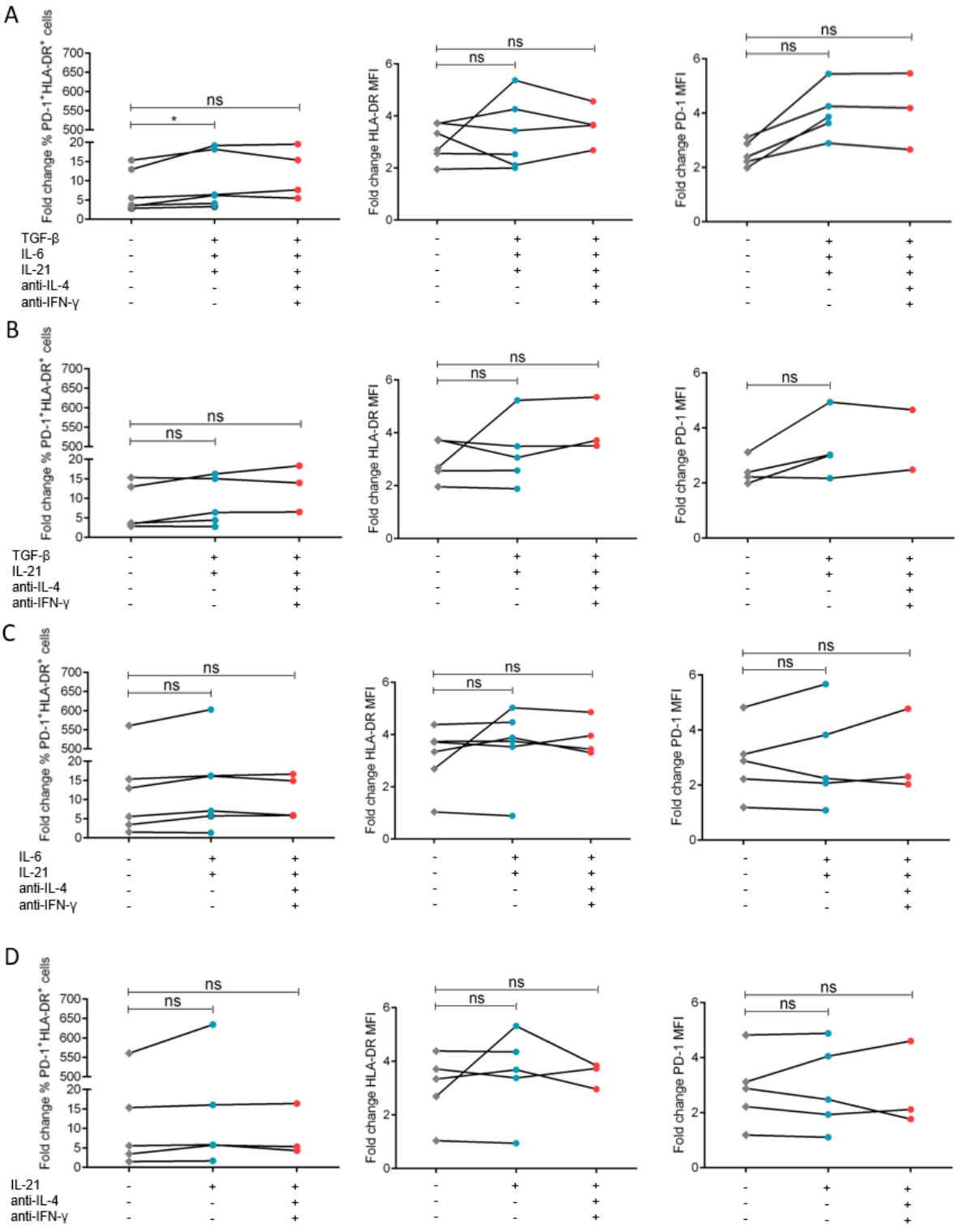


Figure III.4. Decrease in CXCR5 expression after 5 days of cell culture in the presence of 5 µg/mL anti-CD28 and TGF-β and IL-6. Comparison between percentages of CXCR5-expressing PD-1$^+$HLA-DR$^+$ CD4$^+$ T cells before and after differentiation under polarizing conditions. Each data point represents an individual donor and the grey bars represent the medians. Wilcoxon matched-pairs test was used to determine p values (***p < 0.001).

1.4. TGF-β, IL-6 and IL-21 modulate the development of PD-1$^+$HLA-DR$^+$ cells

After selection of anti-CD28 concentration, several cytokine polarizing conditions were tested in order to further direct the differentiation of CD4$^+$ T cells into Tfh-like cells. In exception to a noticeable increase in PD-1$^+$HLA-DR$^+$ cells derived from the medium supplementation with TGF-β, IL-6 and IL-21 (Fig. III.5A), no significant variations were observed in the fold changes of both PD-1$^+$HLA-DR$^+$ cell percentage nor in PD-1 and HLA-DR MFIs, when comparing all simulation conditions in the presence (*grey diamonds*) and absence (*blue circles*) of cytokines (Fig. III.5). Nonetheless, the percentage of PD-1$^+$HLA-DR$^+$ cells tended to increase when cell culture was carried out in the presence of TGF-β plus IL-21 and IL-6 plus IL-21 (Fig. III.5B, C). As to the expression levels of HLA-DR and PD-1, despite non-statistical relevance, greater differences were detected in the expression of PD-1, shown in the values of MFI. The conditions TGF-β+IL-6+IL-21 and TGF-β+IL-21 also lead to an emphasized trend to generate PD-1$^+$HLA-DR$^+$ cells that expressed higher levels of PD-1. Limited differences were obtained from the use of IL-21 alone in comparison with the combination with IL-6 (Fig. III.5C, D), suggesting that IL-6 did not play an impactful role in the polarization of Tfh-like cells. The single use of IL-12, IL-23 and IL-27 in cell culture revealed an absence of relevance for the optimization of Tfh cell *ex vivo* expansion (Fig.

III.5E, F, G). When the neutralizing antibodies to IL-4 and IFN-γ were added to the culture medium, inconsistent and unimpactful results were obtained regarding the percentage of memory $CD4^+$ T cells expressing PD-1 and HLA-DR, and the MFIs of these surface molecules (*salmon circles*, Fig. III.5A, B, C, D, G).



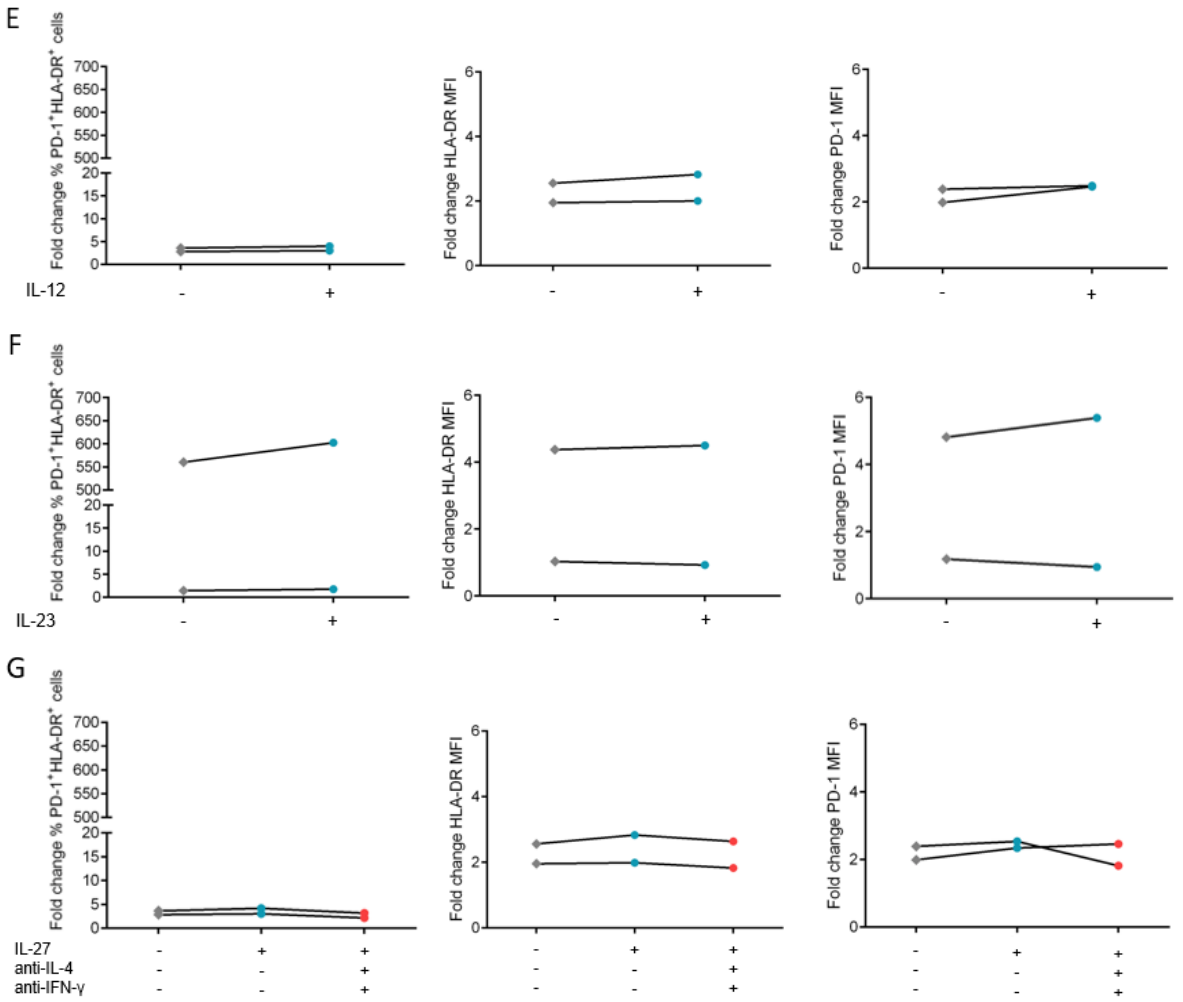


Figure III.5. PD-1$^+$HLA-DR$^+$ CD4$^+$ T cell expansion analysis in different cytokine stimulation conditions. Purified CD4$^+$ T cells were differentiated with 5 µg/mL anti-CD3 and 5 µg/mL anti-CD28 and, when indicated, the cytokine conditions **(A)** TGF-β+IL-6+IL-21, **(B)** TGF-β+IL-21, **(C)** IL-6+IL-21, **(D)** IL-21, **(E)** IL-12, **(F)** IL-23, **(G)** IL-27, in the presence (*blue circles)* or absence *(salmon circles)* of the neutralizing antibodies anti-IL-4 and anti-IFN-γ for 5 days. Plots depict changes in the fold change of PD-1$^+$HLA-DR$^+$ cell percentages (left) and MFI of HLA-DR (middle) and PD-1 (right). Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant; *$p < 0.05$).

Considering that the management of cell culture that recurred to TGF-B, IL-21 and IL-6, or only TGF-B plus IL-21, produced the most striking results in PD-1$^+$HLA-DR$^+$ cell augmentation and in the levels of PD-1 that those cells expressed, these were the polarizing conditions in which the expanded Tfh-like cell population was next investigated in terms of phenotypical and functional characterization.

2. Functional characterization of expanded Tfh-like cells

2.1. PD-1⁺HLA-DR⁺ cells produce cytokines associated with anti and/or proinflammatory actions

Functional analysis of the PD-1⁺HLA-DR⁺ CD4⁺ Tfh-like subpopulation attained comprised the assessment of the cytokine secretion profile, since this feature serves as an indicator of T cell function[99]. Secreted cytokines modulate the function of target cells that express their specific receptors, thus establishing its importance in immune response regulation[100]. To assess intracellular cytokines, isolated CD4⁺ T cells were cultured in the selected proliferation conditions for 5 days. At day 4, cells were re-stimulated with PMA and ionomycin as T cell stimulation with these compounds enhances cytokine production[82]

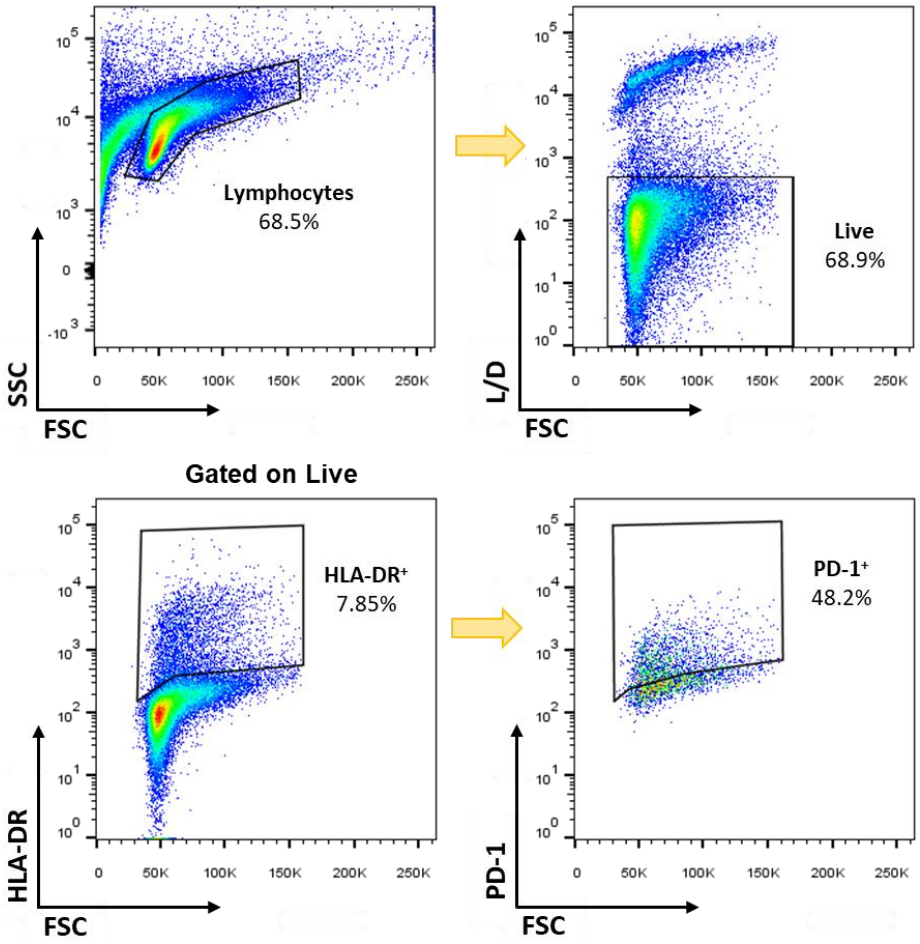


Figure III.6. Representative flow cytometry gating strategy for identification of PD-1⁺HLA-DR⁺ Tfh-polarized cells. For cytokine analysis, upon stimulation under selected polarizing conditions (either TGF-β+IL-21 or TGF-β+IL-6+IL-21) for 4 days, cells were re-stimulated with PMA and ionomycin for 16h. After staining with either Fixable Viability Dye eFluor™ 506 or Fixable Viability Dye eFluor™ 780 and surface labeling, cells were fixed and permeabilized. For transcription factor analysis, fixation and permeabilization was accomplished using the eBioscienceTM Foxp3/Transcription Factor Staining Buffer Set. Intracellular staining was performed with antibodies against the cytokines or transcription factors analyzed. Flow cytometry data was acquired in BD FACS Canto II.

(chapter II, section 2.3). Expanded PD-1$^+$HLA-DR$^+$ T cells were selected as demonstrated in figure III.6. Lymphocytes and live cells were gated as previously described in here. Then the HLA-DR-expressing population was defined and finally, PD-1$^+$ cells were identified. The gating strategy applied does not comprehend the definition of CD4$^+$ cells as purification from PBMCs proved to be effective, confirmed by the evidence that most live cells corresponded to CD4$^+$ cells (Fig. III.2). Moreover, PMA was reported to induce CD4 downregulation from the surface of T cells[101].

Here, a set of cytokines previously reported to be involved in Tfh cell function was evaluated. Tfh cells are defined as IL-21-expressing Th cells and were also shown to have the ability to express IFN-γ, IL-10 and tumor necrosis factor alpha (TNF-α)[87,102-104]. All intracellular cytokines analyzed were expressed at some level, even though no relevant changes were observed in regard to the percentage of cytokine-producing cells when comparing between all three polarizing conditions tested (Fig. III.7A, C; Fig. III.8A, C; Fig. III.9A, C and Fig. III.10A, C). Yet, the stimulation condition in which cell culture was proceeded without additional polarizing cytokines (TGF-β, IL-6 and IL-21; *grey circles*) produced the highest levels of cytokine expression. Differences in the values of MFI between conditions did not show statistical significance either (Fig. III.7B, C; Fig. III.8B, C; Fig. III.9B, C and Fig. III.10B, C), confirming a similar cytokine profile shared by every Tfh-like-polarized population obtained. Regardless of the existence of one outlier and the stimulation condition in question, a great percentage of PD-1$^+$HLA-DR$^+$ cells expressed IL-21, with a median value of 71.48% for the highest value (Fig. III.7C, left panel, *grey bar*).

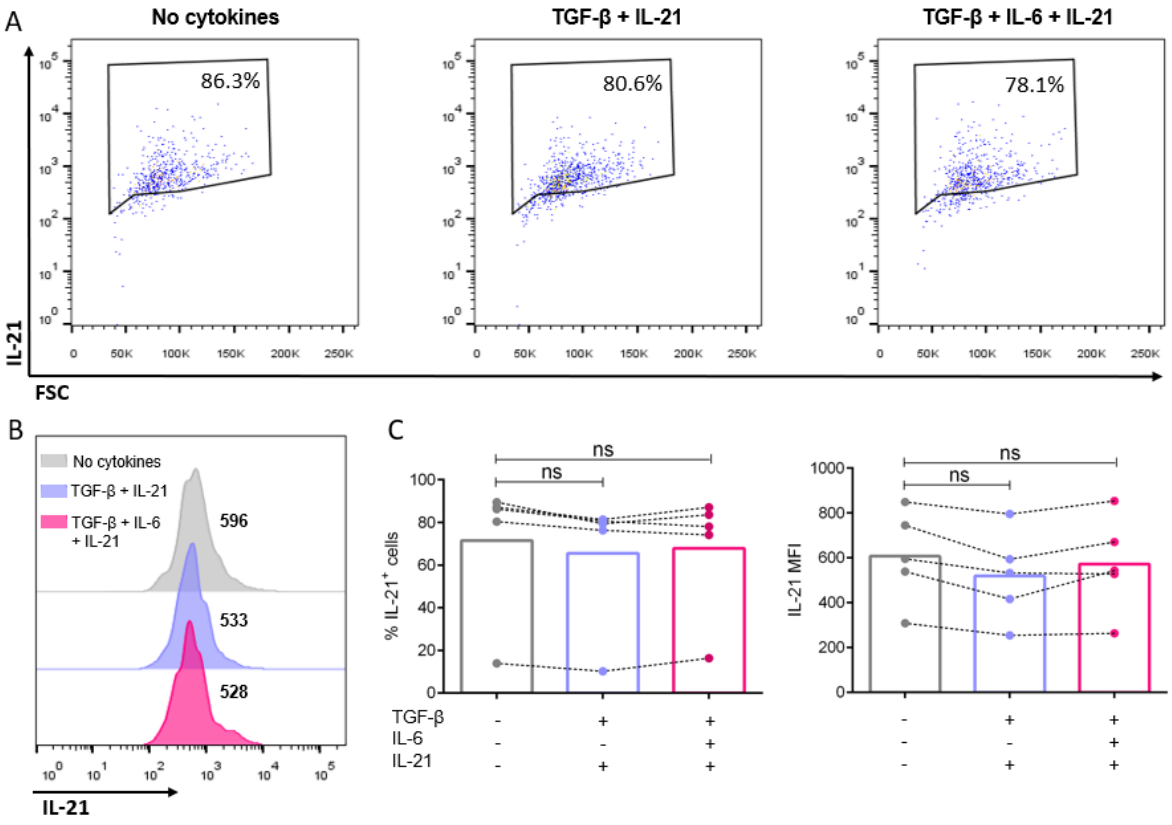


Figure III.7. Flow cytometry analysis of IL-21 production in expanded Tfh-like cells. (A) Representative dot plots of IL-21-producing PD-1$^+$HLA-DR$^+$ Tfh-like cells and **(B)** representative histograms comparing levels of IL-21 expression between the three stimulation conditions indicated. **(C)** Frequency of IL-21$^+$ cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

Without an apparent trend, as depicted in Fig. III.8C (*grey bar*), IFN-γ-producing cells also displayed a relatively high percentage median value (74.98%). Meanwhile, only intermediate levels of TNF-α$^+$ cells were detected with a median value of 54.88% (Fig. III.9C, left panel, *grey bar*). IL-10 presented the greatest differences in the percentage of expressing cells, with the highest median value corresponding to 50.84% while the lowest one, concerning cell culture in the presence of TGF-β, IL-6 and IL-21, corresponded to 36.3% (Fig. III.10C, left panel, *grey and pink bars*).

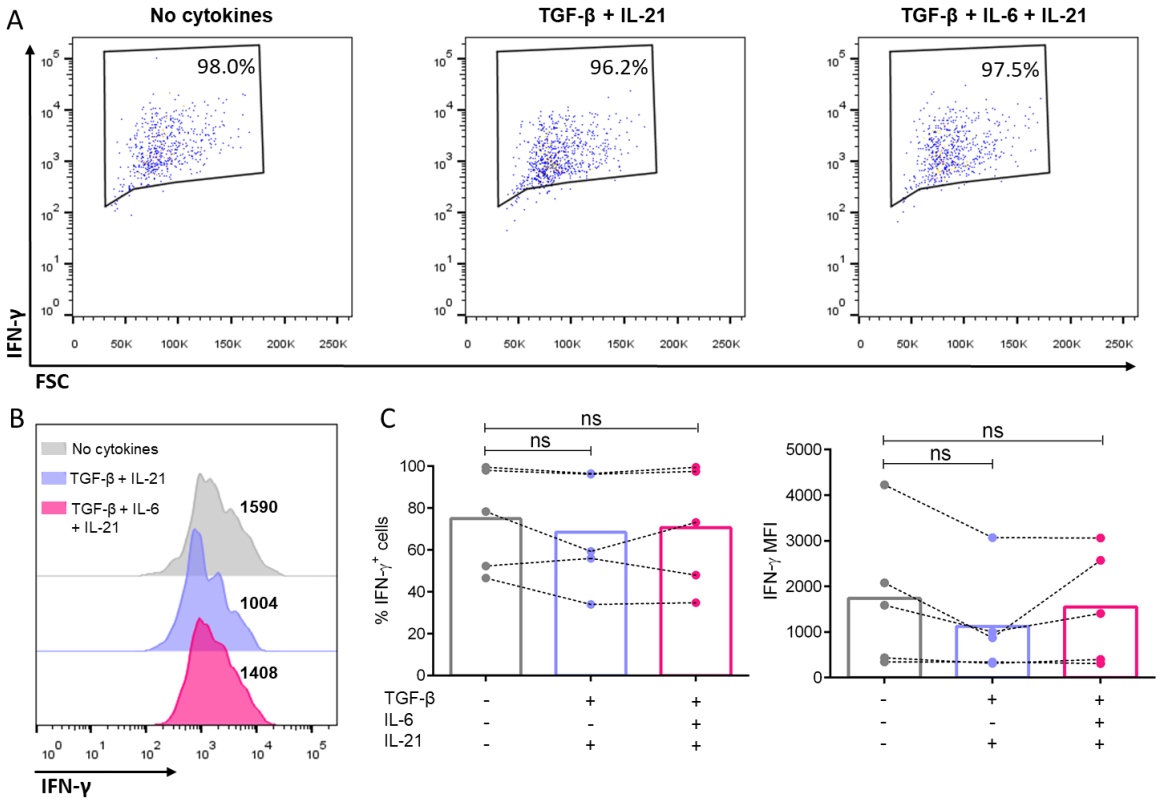


Figure III.8. Flow cytometry analysis of IFN-γ production in expanded Tfh-like cells. (A) Representative dot plots of IFN-γ-producing PD-1$^+$HLA-DR$^+$ Tfh-like cells and **(B)** representative histograms comparing levels of IFN-γ expression between the three stimulation conditions indicated. **(C)** Frequency of IFN-γ$^+$ cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

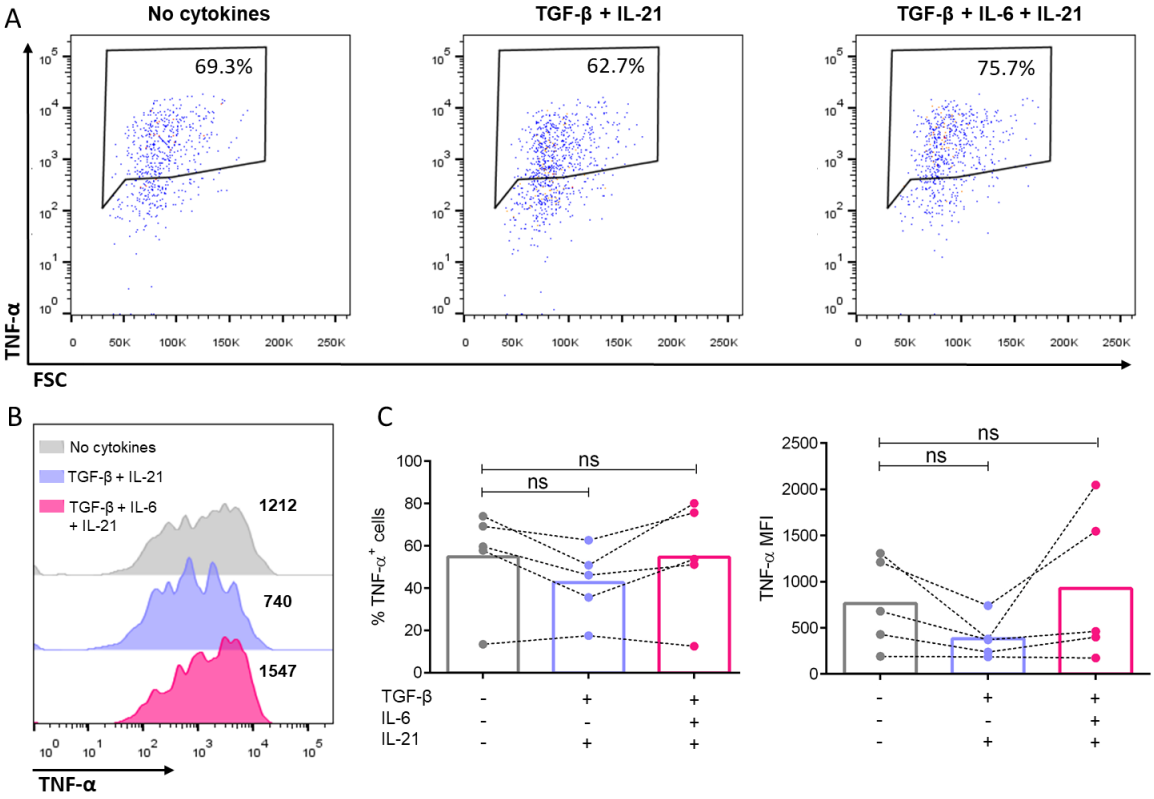


Figure III.9. Flow cytometry analysis of TNF-α production in expanded Tfh-like cells. (A) Representative dot plots of TNF-α-producing PD-1^{+}HLA-DR^{+} Tfh-like cells and **(B)** representative histograms comparing levels of TNF-α expression between the three stimulation conditions indicated. **(C)** Frequency of TNF-α $^{+}$ cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

A lack of significant dissimilarity among the three polarizing conditions, suggests a hypothesis where all PD-1^{+}HLA-DR^{+} Tfh-like cells generated exert equivalent functions. Nonetheless, this affirmation could not be validated without supplementary assessment of cell functionality to further characterize these T helper-like cells.

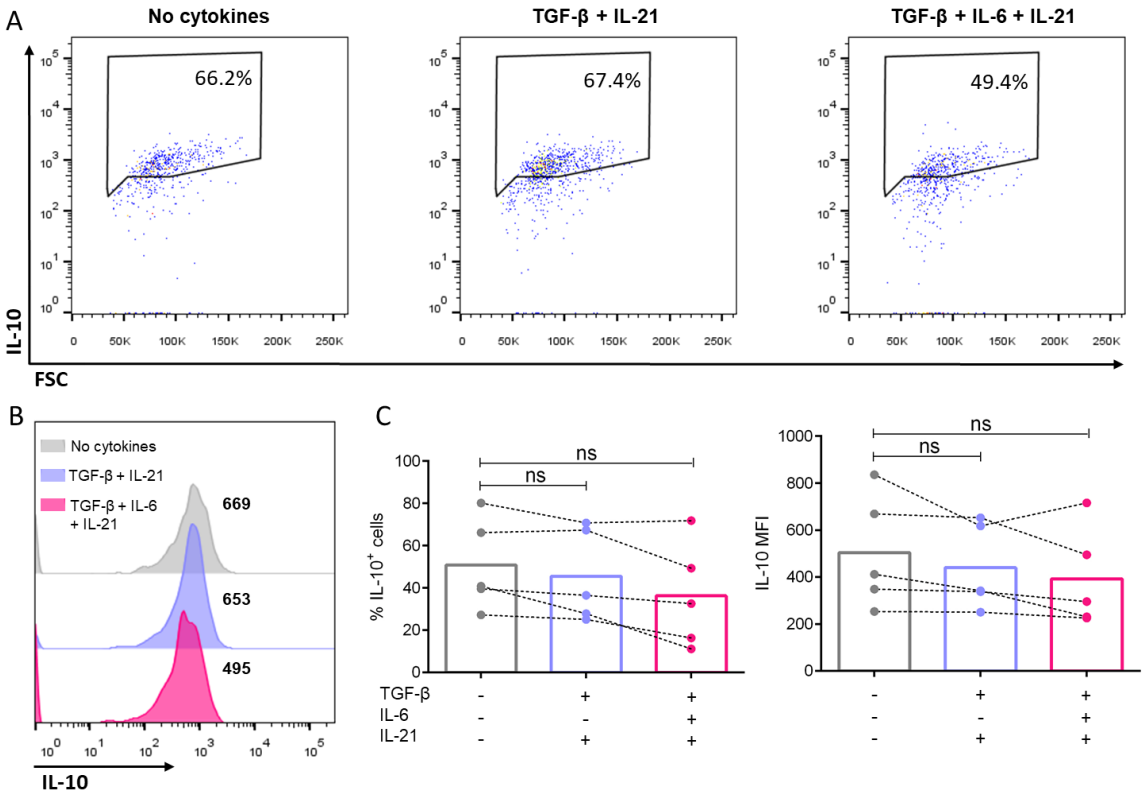


Figure III.10. Flow cytometry analysis of IL-10 production in expanded Tfh-like cells. (A) Representative dot plots of IL-10-producing PD-1$^+$HLA-DR$^+$ Tfh-like cells and **(B)** representative histograms comparing levels of IL-10 expression between the three stimulation conditions indicated. **(C)** Frequency of IL-10$^+$ cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

2.2. Expanded Tfh-like cells are characterized by high Bcl-6 and T-bet expression and contrasting low Foxp3 production

Cytokine secretion patterns specify T cell function in terms of inflammatory and immune responses, while expression of transcription factors within cells determines both their function and distinct lineage classification[105,106]. This classification is usually based on the expression of a specific master transcription factor, Bcl-6 in the case of Tfh-like cells[30]. However, it is the concomitant expression of several transcription factors that explains the functional heterogeneity of CD4$^+$ T cells[106]. Recent work identified several Tfh-derived cell populations able to express T-bet and Foxp3[69,74,103,107,108]. To further functional and phenotypically define the developed Tfh-like populations, gated as demonstrated in figure III.6, functional analysis relative to expression of the mentioned transcription factors was performed. Again, the stimulation condition carried out in the absence of the selected

polarizing cytokines (TGF-β, IL-6 and IL-21; *grey circles*) yielded the highest frequencies of cytokine-producing cells, albeit with no significant variations being observed between conditions. As expected, considerable levels of Bcl-6$^+$ cells were observed (Fig. III.11A, C), with the highest median value being 80.78% (Fig. III.11C left panel, *grey bar*).

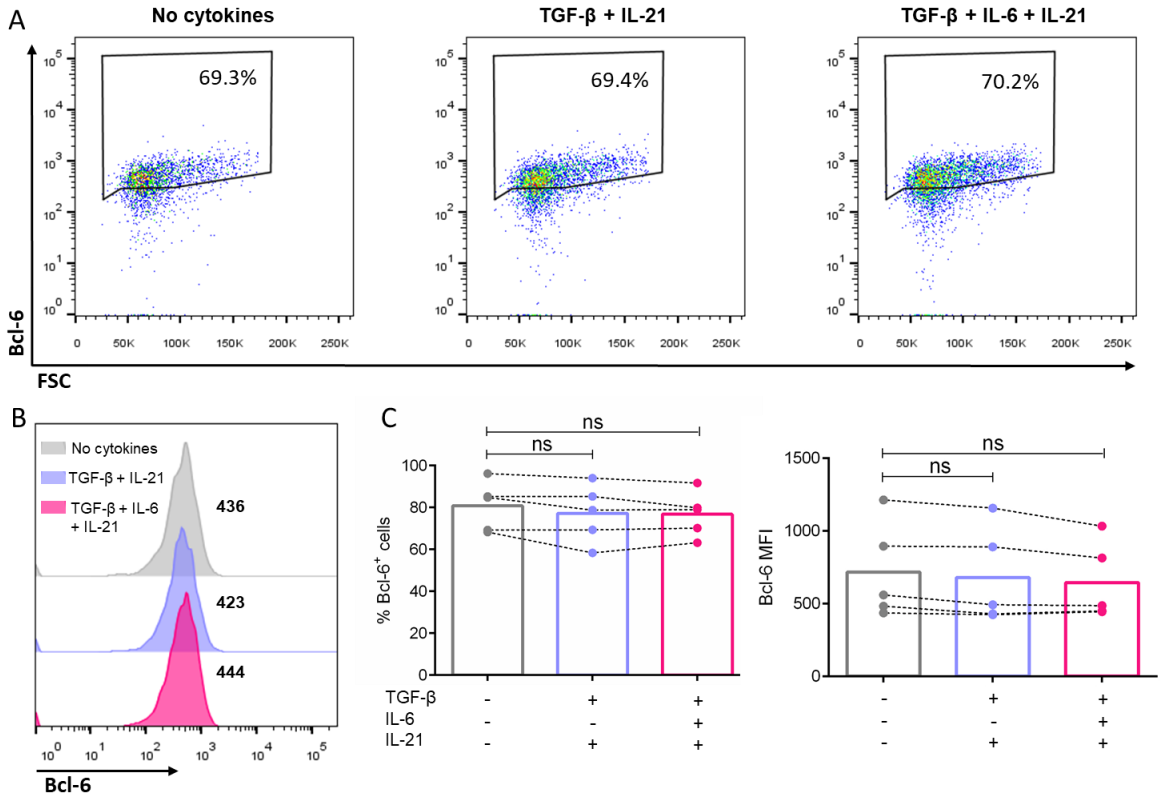


Figure III.11. Flow cytometry analysis of Bcl-6 expression in expanded Tfh-like cells. (A) Representative dot plots of Bcl-6-producing PD-1$^+$HLA-DR$^+$ Tfh-like cells and **(B)** representative histograms comparing levels of Bcl-6 expression between the three stimulation conditions indicated. **(C)** Frequency of Bcl-6$^+$ cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

T-bet was equally highly expressed as determined by the frequency of T-bet$^+$ cells (Fig. III.12A, C), and established in the highest median value of 75.06% (Fig. III.12C left panel, *grey bar*). Concurrently, Foxp3 expression was practically undetectable in some cases, with fewer than 15% cells displaying a Foxp3$^+$ phenotype (Fig. III.13A, C). The lowest variations between conditions were found in the expression of this transcription factor, as confirmed by median values (Fig. III.13 C, left panel). Measured MFI levels of all three transcription factors confirm the inexistence of statistical relevance in observed changes among every stimulation condition tested (Fig. III. 11B, C, Fig. III. 12B, C, Fig. III. 13B, C).

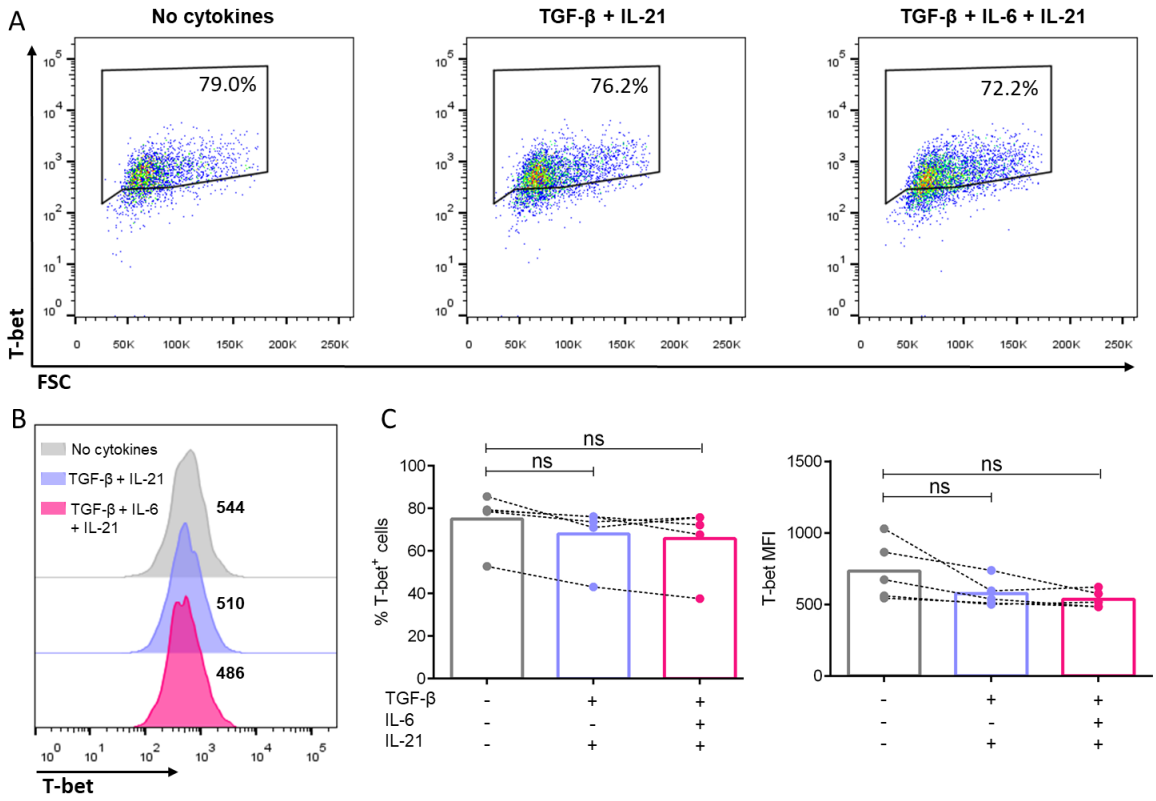


Figure III.12. Flow cytometry analysis of T-bet expression in expanded Tfh-like cells. (A) Representative dot plots of T-bet-producing PD-1^{+}HLA-DR^{+} Tfh-like cells and **(B)** representative histograms comparing levels of T-bet expression between the three stimulation conditions indicated. **(C)** Frequency of T-bet^{+} cells **(**left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

In the assessment of the cytokine profile it was observed that the stimulation condition with the second highest median frequency of cytokine-producing cells was that of TGF-β and IL-21. Contrary to that, concerning expression of the transcription factors examined, the second highest percentage values were obtained when Tfh-like-polarized cells were cultured in the presence of all three cytokines TGF-β, IL-6 and IL-21. Still, an absence of statistical significance found in the collected results reinforce the idea that irrelevant differences between conditions translate into a single Tfh-like signature with the same functional activity.

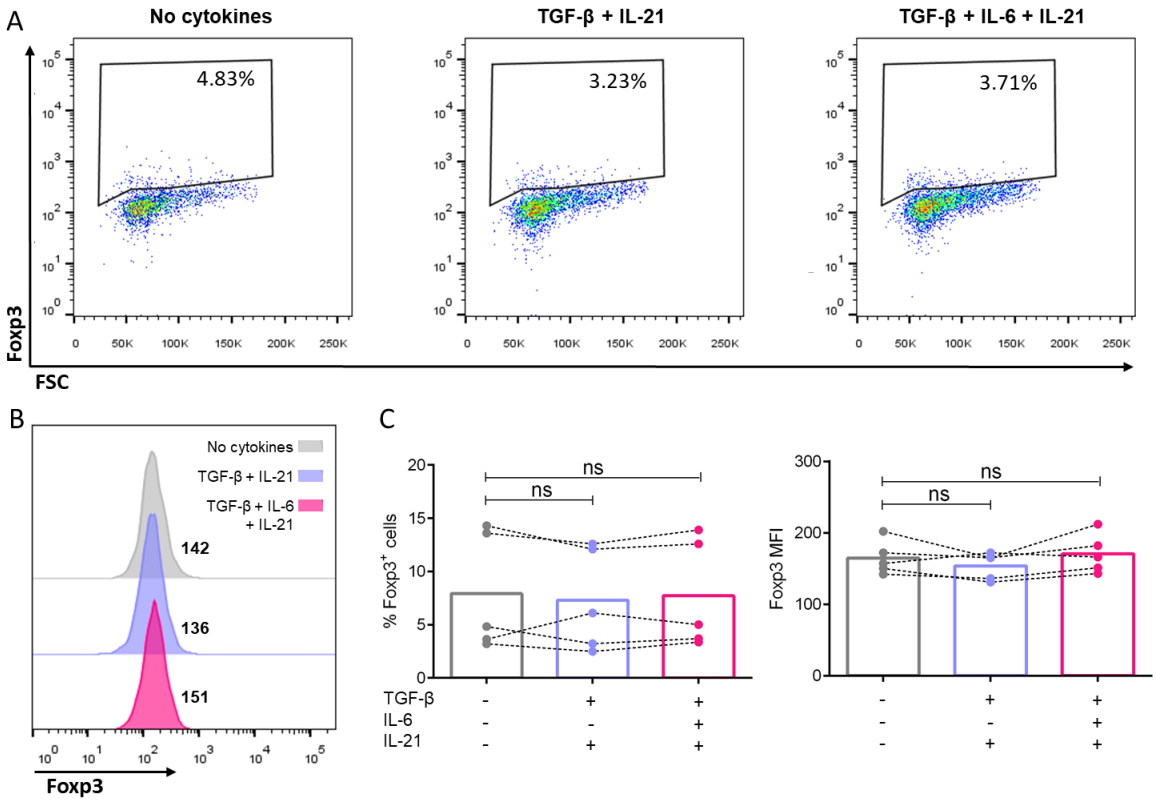


Figure III.13. Flow cytometry analysis of Foxp3 expression in expanded Tfh-like cells. (A) Representative dot plots of Foxp3-producing PD-1^{+}HLA-DR^{+} Tfh-like cells and **(B)** representative histograms comparing levels of Foxp3 expression between the three stimulation conditions indicated. **(C)** Frequency of Foxp3^{+} cells (left) and respective MFI (right). Grey bars represent the medians in the absence of cytokines, blue bars represent the medians in the presence of TGF-β and IL-21, while pink bars represent the medians in the presence of TGF-β, IL-6 and IL-21. Each data point represents an individual donor. Statistical analysis was performed using Wilcoxon matched-pairs test (ns: not significant).

CHAPTER IV • **DISCUSSION**

Follicular helper T cells play an essential role in humoral immunity due to their B cell helper activity, ensuring an adequate antibody production and providing an effective protection against infection or vaccine-induced immunity challenge[17,109]. An impressive amount of work has reported associations between Tfh-like cells and several diseases[24]. Despite the efforts to assess Tfh cell function in illness conditions, an insufficiency of knowledge remains an impediment to define the precise role of these T cells and their adjacent clinical significance. Specifically, in rheumatoid arthritis and breast cancer, recently found $CXCR5^{-}PD\text{-}1^{hi}$ $CD4^{+}$ T cells were attributed pathogenic and defensive roles[79], enhancing the lack of understanding regarding Tfh-like cell subpopulations found expanded in unhealthy individuals. In the present study, $PD\text{-}1^{+}HLA\text{-}DR^{+}$ $CD4^{+}$ T cells were expanded *ex vivo* under inflammatory conditions with twofold objectives. First, to determine adequate proliferation conditions of these resembling-Tfh cell populations found in certain types of cancer and inflammatory diseases and second, to functionally characterize expanded cells and confirm the expression of a Tfh-like gene signature.

To achieve this, differentiation of healthy human $CD4^{+}$ T cells isolated from blood samples was performed. Cell culture was managed in the presence of the cytokine IL-2. It has been demonstrated that excess IL-2 comprises the maintenance or survival of T cells following the Tfh cell differentiation pathway, suppressing this process[44]. Nonetheless, the service of this cytokine as an initial boost for potent T cell expansion *in vitro* has also been reported[110]. In agreement to the latter, in this work, when cell culture was ensued in the absence of IL-2 the lymphocyte population could not be identified as cells were not alive (data not shown), suggesting that T cells did not survive for 5 days in a medium deficient in IL-2.

As it was shown that increasing concentrations of anti-CD28 in combination with a high concentration of anti-CD3 could increase T cell activation[97], various anti-CD28 concentrations were tested in order to define proper proliferation conditions for Tfh-like cells to develop. An increase in the levels of expression of PD-1 exhibited by memory $PD\text{-}1^{+}HLA\text{-}DR^{+}$ $CD4^{+}$ T cells with the addition of TGF-β and IL-6 to the culture medium, two cytokines demonstrated to be relevant for Tfh differentiation, as highlighted in section 2.2 of chapter I, in combination with a slight increment in the percentage of these cells (Fig. III.3), were determining factors for selecting the concentration of 5 µg/mL for anti-CD28.

Previous studies reported the scarcity of memory $CXCR5^{+}$ $CD4^{+}$ T cells in the peripheral blood of healthy individuals and the identification of $CXCR5^{-}$ Tfh-like cell populations within non-lymphoid inflamed tissues[78]. Accordingly, upon expansion $PD\text{-}1^{+}HLA\text{-}DR^{+}$ T cells displayed a decrease in CXCR5 in comparison to baseline $PD\text{-}1^{+}HLA\text{-}DR^{+}$ T cells identified before cell culture (Fig. III.4). The downregulation of this chemokine receptor comes as no surprise since it is related to B cell follicle homing of Tfh cells within the GC[111].

In addition to CD3 and CD28 stimulation and the use of IL-2, several cytokine stimulation conditions were tested (Fig. II.1). When $CD4^+$ T cells were cultured in the presence of TGF-β and IL-21 or TGF-β, IL-6 and IL-21, a slightly more noticeable expansion of $PD\text{-}1^+HLA\text{-}DR^+$ cells was observed as well as a trend for increased levels of PD-1 expression when compared to the conditions in the absence of these cytokines. Despite having an n=2 for the experiments in which IL-12, IL-23 and IL-27 were individually used, no relevant results indicated an advantage of the single use of these cytokines. In future studies, different combinations of these proteins with the selected polarizing cytokines (TGF-β, IL-6 and IL-21) should be conducted to further investigate the optimal proliferation conditions to direct Tfh cell differentiation and optimize *ex vivo* expansion of Tfh-like cells.

To confirm a Tfh-like resemblance and functionally characterize the *ex vivo* expanded $PD\text{-}1^+HLA\text{-}DR^+$ $CD4^+$ T cells, the cytokine secretion profile and expression of transcription factors were assessed. The production of IL-21, IFN-γ, IL-10 and TNF-α, with different levels of expression, was confirmed (Fig. III.7C, 8C, 9C and 10C). The absence of statistical relevance concerning the different percentages of cytokine-producing cells obtained among all stimulation conditions applied, indicates that a single gene signature sharing identical functionality was produced. This was then confirmed by the lack of relevance also observed in the results obtained regarding the expression of the transcription factors Bcl-6, T-bet and Foxp3 (Fig. III.11C, 12C, 13C).

A previous study revealed a role for IL-6 in triggering the production of IL-21 by human Bcl-6-expressing $CD4^+$ T cells[112]. That occurrence was not replicated in this work, as Tfh-polarization derived from cell culture in the presence of TGF-β, IL-6 and IL-21 did not develop relevant increased frequencies of $IL\text{-}21^+$ cells, when compared to results obtained from cells cultured only with TGF-β and IL-21. In agreement to these data, a recent review claimed that the role of IL-6 in human Tfh cell differentiation is yet to be understood[24]. Another work reported that, in an IL-2-dependent manner, TGF-β induced *in vitro* expression of Foxp3 in $CD4^+$ T cells[113]. Although in low amounts, $PD\text{-}1^+HLA\text{-}DR^+$ Foxp3-producing cells were identified. However, similar frequencies of $Foxp3^+$ cells were observed when $CD4^+$ T cells were cultured in medium lacking TGF-β.

IFN-γ-expressing GC Tfh cells that previously expressed the transcription factor T-bet were identified[103]. Expression of IFN-γ in $CD4^+$ T cells is regulated by T-bet[80], which might explain the relatively high levels of $IFN\text{-}\gamma^+$ and $T\text{-}bet^+$ cells that were found within the Tfh-like cell population described in the present work. Hence, these cells exhibit phenotypical similarities with cTfh1 cells, excluding the reduced IL-21 expression found in this circulating Tfh counterpart[63]. A higher production of IL-21 could indicate an enhanced B helper activity in the expanded Tfh-like cell population, as this typical Tfh cytokine commonly associated with B cell help was suggested to significantly influence B cell function regulation *in vivo*[114,115]. Moreover, Tfh-secreted IL-21 has also been associated with participating roles in autoimmune diseases. For example, in rheumatoid arthritis, this cytokine was correlated with plasma B cell proliferation and maturation and, consequently, production of autoantibodies[102,116]. Taking these data into account, it is plausible to speculate that the

expanded Tfh-like cell subpopulation studied in this work might play a pathogenic role in rheumatoid arthritis in similarity with T_{PH} cells identified in Rao, D. A. *et al.* (2017) and before-mentioned here. In agreement with this hypothesis, the expression of TNF-α, one of the key cytokine molecules that causes inflammation in this autoimmune disease[117], was detected at intermediate levels. On the other hand, due to its antitumor effects, administration of TNF-α has been included in cancer treatment for patients suffering from soft tissue sarcoma confined to the limb and metastatic in-transit melanoma[118]. Furthermore, intermediate frequency levels of IL-10$^+$ cells were found within the expanded cell subset, as described for T_{FH}X13 cells characterized in Gu-Trantien, C. *et al.* (2017). This cytokine is predominantly linked to anti-tumor properties and its tumor inhibiting activity has been implied to take place in breast cancer[119]. Thus, Tfh-polarized cells expanded in this work could also exert protective functions in this type of cancer.

As earlier evidenced, it was reported that T_{PH} cells were related to T_{FH}X13 cells. Even though these two cell populations were associated with contradicting roles in rheumatoid arthritis and breast cancer, the phenotypical resemblances between them are undeniable, as portrayed in figure I.5. Furthermore, both T_{PH} and T_{FH}X13 cells are linked to ectopic (or tertiary) lymphoid structures (ELS), for the chemokine CXCL13 was expressed at high levels, and development and maintenance of ELS generally demand the presence of CXCL13 and B cells[120]. Then, these cells may defensively or pathogenically influence the diseases they have been associated with by recruitment of other immune cells, as consequence of ELS formation and, in the case of rheumatoid arthritis, they also may support autoantibody production by B cells[24].

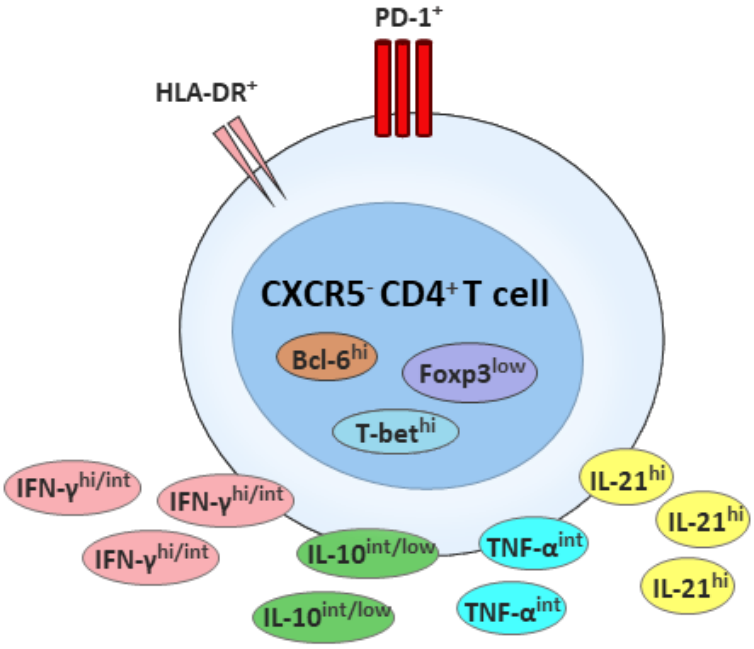


Figure IV.1. Tfh-like cells expanded *ex vivo* from blood samples. PD-1$^+$HLA-DR$^+$ T cells displaying phenotypical similarities, as discovered by measurement of cytokine or transcription factor-producing cells, to recently described protective T_{FH}X13 and pathogenic T_{PH} cells, found in breast cancer and rheumatoid arthritis, respectively.

Altogether, these data suggest that the subset of PD-1^{+}HLA-DR^{+} CD4^{+} T cells developed in the present work, could potentially exert protective or pathogenic functions in cancer and rheumatoid arthritis, respectively, due to phenotypical correspondences to the Tfh-like cell subpopulations found in those diseases (Fig IV.1). The mechanisms through which this diversion of function occurs remain unknown. Still it is reasonable to hypothesize that these mechanisms could depend on, for example, microenvironment signals such as cytokines and the presence of certain immune cells. As discussed here, these other cells could be recruited by Tfh-like cells if ELS are being formed in diseased tissue. Upon influence of these signals, Tfh-like cells might adapt to them and therefore compromise to either defensive or detrimental roles. Lastly, this work demonstrated that *ex* vivo expansion of Tfh-like cells facilitates the study of novel subpopulations that arise in several disease conditions.

CHAPTER V • **CONCLUSION AND FUTURE PERSPECTIVES**

With the principal aim of inducing *ex vivo* expansion of Tfh-like cells, the present work served its purpose as PD-1$^+$HLA-DR$^+$ CD4$^+$ T cells displaying expression of the Tfh markers Bcl-6 and IL-21 were obtained. Nonetheless, it is highly likely that the factor that lead to the generation of these cells was the stimulation of CD3 and CD28, for no cytokine stimulation condition tested significantly distinguished themselves from cell culture in the absence of any additional cytokine (excluding IL-2). One explanation for this could be that the 5 days established for cell culture were not enough for CD4$^+$ T cells to completely conclude differentiation, given that it was suggested that *in vivo* complete Tfh cell differentiation occurs over a period of nearly 10 days[121]. Opposed to this, *in vitro* naive CD4$^+$ T cell directing into Tfh-like subset was successfully conducted for 3.5 days[85]. Thus, to overcome this system limitation different cell culture protocols should be tested, particularly to study the influence of time and the use of alternative cytokine combinations, as discussed in Chapter IV, with the goal of optimizing *in vitro* Tfh-like cell polarization.

Despite the fact that the expanded Tfh-like subpopulation appeared to exhibit functional similarities with both T_{PH} cells and $T_{FH}X13$ cells, further phenotypical analysis should be carried out in future studies to properly confirm whether generated cells are more prone to play a protective or pathogenic role. With a defensive activity associated, *ex vivo* expansion could consist in a useful tool to induce *in vivo* expansion in certain types of cancer and ultimately be included in the development of a novel cancer immunotherapy. On the other hand, linked to pathogenic roles, this approach grants a valuable research methodology to expand the knowledge regarding cell populations emerging from inflammatory diseases, such as rheumatoid arthritis or SLE. In this spectrum of the situation, a deeper understanding of these populations associated with disease pathogenesis and/or negative prognosis could eventually culminate in the development of new treatment strategies targeting Tfh-like cells.

Overall, considerable advances in this field have been attained over the past few years, though, as depicted in the present study, Tfh cell biology is yet to be fully understood in the interest of applying that knowledge to public health needs.

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